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The Behavioral Neuroendocrinology of Fish Sex Change: The Role of Steroids and Monoamines

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THE BEHAVIORAL NEUROENDOCRINOLOGY OF FISH SEX CHANGE: THE ROLE OF STEROIDS AND MONOAMINES

by

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Under the Direction of Matthew S. Grober

ABSTRACT

Social status influences reproductive physiology in many species, and sex change in marine teleost fishes provides an excellent model to understand how an organism can modulate its reproductive system in response to social stimuli. The series of experiments presented in this dissertation has focused on the proximate mechanisms underlying sex change and, in particular, the neuroendocrine factors that might translate social information into physiological changes. The bluebanded goby (*Lythrypnus dalli*) is a sexually plastic fish, and the dominant female typically changes sex when the male is removed from the social group. The direct physical interactions between the male and the females were found to be the main sensory cues that inhibit sex change. Sex steroids can both modulate and be modulated by behavior, and as a result they have been the most obvious candidates for a key role in the regulation of sex change. Males and females showed similar diurnal patterns for steroid hormones, but females had significantly higher water-borne

estrogen levels. Concentrations of estradiol, testosterone and 11-ketotestosterone presented sex and tissue differences in brain, gonad and muscle, and they varied in complex ways in different tissues during sex change. The neurotransmitter serotonin (5-HT) has been suggested to be involved in the inhibition of socially regulated sex change because of its role in the modulation of both reproductive and aggressive behavior. None of the pharmacological manipulations performed in *L. dalli* to alter serotonergic activity was able to overcome the input from the social environment and affect sex change. Neither monoamine levels nor the area or number of 5-HT immunoreactive neurons were different between males, females and sex changers or between dominant and subordinate females. The results do not support the hypothesis of a serotonergic inhibition on sex change in *L. dalli*, but show that rapid changes in brain androgen levels might be implicated in inducing behavioral or morphological changes associated with sex reversal. Also, steroids respond to changes in the social environment in different ways in different tissues so local steroid synthesis should receive greater attention, and caution is required when using circulating levels to understand behavioral regulation.

INDEX WORDS: Sex change, Steroids, Serotonin, Social status, Protogynous, Diurnal patterns, Brain, Gonad, Goby

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DEDICATION

To my parents,

Thank you for letting me follow my passions and never holding me back.

Without your wonderful example on how to deal with life, and your incredible support and love throughout every step of this journey, I would never be where I am and who I am.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Social interactions are an important evolutionary force in generating phenotypic variation because behavioral interactions can affect the development and maintenance of inter-individual variation (Stamps, 2003) and these interactions are much more common and complex in highly social species. The flourishing field of epigenetics provides many examples of how social environment can have significant physiological impacts by influencing the relationship between genotype and behavior during sensitive developmental periods (reviewed by Crews 2008). The social environment can also have physiological effects during adulthood and these effects are particularly pronounced during life history transitions (Taborsky, 2001**).**

Social Interactions and Reproductive System

Dominance interactions are a type of social interaction that affects most social animals and their outcome is very important because it produces differential access to limited resources (food, territories, shelter, status, and mates) and dramatically affects their reproductive success (reviewed by Ellis, 1995). Social interactions can also exert potent effects directly on the reproductive system. For example, inhibition of reproductive behavior and/or function in subordinate individuals is documented in many taxa from insects (honeybees: Keller and Nonacs, 1993; Winston and Slessor, 1992), to fish (Poeciliidae: Borowsky, 1987; Cichlidae: Francis et al., 1993), to mammals (dwarf mongooses: Creel et al., 1992; naked mole-rats: Clarke and Faulkes, 2001). Sensory transduction of social stimuli regulates a cascade of neuroendocrine events leading to behavioral, morphological, and physiological transformations that can, in

extreme cases, end up in complete inhibition of reproductive function but the specific mechanisms are not known yet.

An interesting example of the interaction between social environment and reproductive function are species that exhibit socially regulated sex change, because the reproductive function of one sex is inhibited while the one of the other sex is activated. Many invertebrates and fishes are capable of socially regulated sex change, whereby aspects of the social environment are critical for inhibiting and/or initiating the transition from one sex to the other (Francis, 1992; Godwin et al., 2003; Warner et al., 1996; Warner and Swearer, 1991). In protogynous hermaphroditic fishes, where females can change sex into males, dominant males can inhibit lower-ranking females from changing sex simply by maintaining a stable social hierarchy (Rodgers et al., 2007). The removal of a male from the social group relaxes the hierarchy and induces a female, usually the dominant female, to change sex (Reavis and Grober, 1999). This sexual plasticity or ability to switch to a different sexual phenotype depending on environmental circumstances (Bass and Grober, 2009) increases the reproductive success of the individual by increasing the opportunity for finding or monopolizing mates. This is particularly true for the sex changer, since many protogynous males spawn with multiple females (St. Mary, 1994).

In mammals, sex is determined by genes that regulate early gonadal development, and the gonad then produces steroids that feminize or masculinize a variety of body tissues (Phoenix et al., 1959), including the brain (Gorski et al., 1980). In hermaphroditic fishes on the other hand, the signal responsible for the initiation of sex reversal most likely starts in the form of a change in the brain neuroendocrine milieu (Grober and Bass, 1991), because the change in the social environment must be processed by the central nervous system first, and then the information is transmitted throughout the body. Once the gonads start to transform and change their steroid

production, they can provide feedback to the brain and body to orchestrate the many changes in tissue and organ function (Devlin and Nagahama, 2002). Work on the protogynous bluehead wrasse *Thalassoma bifasciatum* shows that the gonads are not needed for the initiation of sex change, because gonadectomized females can show color and behavioral changes typical of fish becoming males (Godwin et al., 1996). It remains unclear how the social cues are translated into the physiological changes that eventually transform the gonad and the rest of the body.

This need to translate social cues into physiological signals is not only pertinent to sex changing fish but to any social animal including humans. To maximize their reproductive success and survival, animals need to process social information and make the appropriate decision (Insel and Fernald, 2004). Therefore, understanding the neuroendocrine mechanisms underlying sex change provides valuable information applicable to many fundamental behavioral processes.

Sensory Cues

Although many types of sensory cues might be responsible for regulating socially controlled sex change, few studies have examined the relative importance of different sensory cues as proximate mechanisms involved in the inhibition of protogynous sex change (Cole and Shapiro, 1995; Ross, 1981; Ross et al., 1983; Shapiro, 1983). Females might perceive the presence or absence of a male through visual, chemical or tactile cues, or a combination thereof. Visual stimuli from a territorial male can affect the reproductive decisions of females. For example, in the saddleback wrasse *Thalassoma duperrey*, visual cues typical of the territorial terminal phase male phenotype (e.g., coloration patterns) can inhibit female sex change and stimulate ovarian function (Morrey et al., 2002). Chemical signals also play an important role in fish reproductive

behavior (Liley, 1982; Liley and Stacey, 1983) and could modulate the process of sex change. For instance, in the goby *Coryphopterus glaucofraenum*, chemical cues emitted from conspecific females increased the rate of sex change in solitary females (Cole and Shapiro, 1995). Tactile stimuli, in the form of physical interactions during agonistic encounters, can prevent sex change in females of territorial species (Fricke and Fricke, 1977; Lutnesky, 1994; Robertson, 1972; Shapiro, 1981a). In my first chapter, I will critically examine the contribution of visual, olfactory and tactile cues in the assessment of male presence by females. In the remaining chapters, I will examine the changes in neurotransmitters and steroids that result from that assessment and participate in the transduction of social cues into physiological signals. I will focus my attention on the steroid hormones estradiol, testosterone and 11-ketotestosterone (Chapters 2 and 3), and the monoamine serotonin (Chapters 4 and 5).

Steroid Hormones

In fish, as in mammals, circulating levels of 17β-estradiol (E2) are typically higher in females than males, and E2 is considered essential for ovarian development and maintenance by acting directly on oocytes and indirectly on other tissues (e.g., vitellogenin production in the liver, Devlin and Nagahama, 2002). In contrast to most mammals, in many fish there is no sex difference in testosterone (T) concentrations and in some, females have higher T levels than males (Borg, 1994). In the ovary, T is produced in the thecal layer of the follicle and is converted by aromatase into E2 in the granulosa layer (Devlin and Nagahama, 2002), so elevated levels of T in females are probably needed to maintain a high rate of E2 synthesis. The hormone 11-ketotestosterone (KT) is considered the primary male androgen in fish and is critically involved in testicular differentiation (Devlin and Nagahama, 2002). Both KT and its precursor T are

produced in the Leydig cells of the testis. KT has a strong masculinizing effect and implanting KT can induce development of testes even in females of gonochoristic species such as goldfish (Kobayashi et al., 1991). In gonochoristic species and in some hermaphrodites, females typically present very low or undetectable levels of KT and males have high circulating levels (Borg, 1994; Devlin and Nagahama, 2002). It is interesting to note that in some hermaphroditic fishes there are no sex differences in KT levels or males have levels much lower than in gonochoristic species (Borg, 1994) so these low KT levels might be associated with the presence of adult sexual plasticity (Kroon et al., 2003).

While many studies examined changes in steroids during sex reversal, most of them focused on circulating or whole body homogenate levels. The gonads are certainly an important source of steroids and they probably make a major contribution to plasma steroid levels, but other sites of steroidogenesis for androgens (Mayer et al., 1990b; Schulz, 1986; Schulz and Blum, 1991) and estrogens (Andersson et al., 1988; Pasmanik and Callard, 1986) certainly exist. In particular, the brain's ability to produce steroids has generated great interest, and local steroid synthesis might represent a novel feedback interaction between gonad and brain in the control of gonadal development (Devlin and Nagahama, 2002). Also, there is the possibility that what were considered simply as target tissues of steroid action might actually be able to control their own local steroid synthesis (Schmidt et al., 2008). Apart from having a different rate of steroid synthesis or catabolism, different tissues can also have differential expression of steroid receptors, and therefore their sensitivity and the amount of steroids they can bind can vary. In the classical model of action, steroids bind to intracellular receptors and then alter gene expression, but this takes a relatively long time. There is evidence that both estrogens (Woolley, 2007) and androgens (Michels and Hoppe, 2008) can act through fast, non-genomic mechanisms and have

an effect within seconds or minutes, and this time frame fits better with the rapid steroidal responses to behavioral stimuli (Remage-Healey and Bass, 2006). Because of all the reasons mentioned, I believe that circulating levels might not always be an accurate estimate of what happens in specific organs during sex change, so in Chapter 3, I have taken a novel approach for the study of fish behavioral endocrinology by examining steroid levels in specific tissues.

Steroid hormones and social environment

It is common knowledge that the brain controls behavior but it is also the case that behavioral interactions and social information can influence brain structure by affecting brain development and/or inducing changes in the adult nervous system (e.g., gene expression; Insel and Fernald, 2004; Robinson et al., 2008). A very important characteristic of steroid hormones is that their production is responsive to the social environment. In fact, not only can they modulate behavior, but they can be modulated by behavioral interactions. Estrogens and androgens are of course important hormones in the reproductive context, but they are involved in the modulation of other functions such as aggression or social status. For example, in the protogynous stoplight parrotfish *Sparisoma viride*, males had higher T and KT after acquisition of a new territory or after simulated territorial intrusion (Cardwell and Liley, 1991a). In the cichlid *Neolamprologus pulcher*, both males and females increased KT in response to territorial intrusion, and females also increased T (Desjardins et al., 2006). In the African cichlid *Astatotilapia burtoni*, both T and KT are higher in dominant territorial males than in subordinate males (Parikh et al., 2006). In rainbow trout *Onchorhynchus mykiss*, spawning in the laboratory, dominant males had higher plasma T levels than subordinates, and this appears to be a result rather than a cause of their social status because steroid levels increased in subordinate males that became dominant. In free-

ranging brown trout *Salmo trutta* sampled in the field, KT but not T was higher in dominant males (Cardwell et al., 1996) so there are interspecific differences.

Steroid hormones and sex change

Because of their role in sexual differentiation, steroids have been the obvious focus of many studies on sex change. As a very general rule, but with many known exceptions, E2 tends to drop during protogynous sex change, and KT tends to increase, while T increases in some species but not in others (reviewed by Devlin and Nagahama, 2002, and by Frisch, 2004). In the honeycomb grouper *Epinephelus merra*, females treated with an aromatase inhibitor to block E2 synthesis developed testis, and showed higher levels of both T and KT (Bhandari et al., 2005). E2 might directly inhibit KT synthesis or high KT could be a consequence of the development of testis and active spermatogenesis due to lack of E2. In this species, KT is able to induce sex change without manipulating E2; in fact, KT implants transformed female *E. merra* into males and reduced their E2 levels (Bhandari et al., 2005).

It is often hard to compare results from different experiments on sex change because concentrations of steroid levels can vary across different species, and within a species, they can vary dramatically during the course of the year depending on the reproductive season, during the course of the day due to diurnal rhythms and within hours due to social interactions. For example, in males from a natural population of rainbow trout, KT was higher in concentration than T in spawning condition and declined to undetectable levels in post-spawning fish (Liley et al., 1986). Of course all the different factors affecting steroid concentrations can overlap in time, so rigorous studies controlling for all these conditions are needed to be able to separate the effects of social interactions from the rest. When analyzing individual changes in steroids, I have

used only animals during the reproductive season and I have taken into account the diurnal rhythms of hormones (Chapter 2).

Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine that acts as a neurotransmitter and neuromodulator in the central and peripheral nervous system of both vertebrates and invertebrates. In vertebrates, it is produced in enterochromaffin cells in the gastrointestinal tract, in platelets in the blood stream, and in serotonergic neurons in the central nervous system. Serotonin has a huge variety of functions and it can have inhibitory or stimulatory effects depending on the type of receptors it acts on (reviewed for sexual behavior by Hull et al., 2004). Some major illnesses or disorders, including depression and aggressive behavior, are attributed to abnormal brain levels of this neurotransmitter, and common treatments are drugs based on selective serotonin reuptake inhibitors (SSRI) that increase serotonergic activity in the synapse (Vaswani et al., 2003). An established side effect of some of these drugs in human patients is a reduction of sexual function (Montgomery et al., 2002). This monoamine is ubiquitous in the animal world and its involvement in the modulation of aggressive and reproductive behavior is generally conserved within major taxa.

Serotonin and reproduction

There is much experimental evidence supporting an inhibitory role of serotonin on reproductive behavior. High 5-HT levels are associated with decreased sexual behavior in rodents (Hull et al., 2004). Administration of a 5-HT_{1A} receptor antagonist facilitates lordosis in male rats (Kishitake and Yamanouchi, 2004), and the agonist 8-OH-DPAT inhibits lordosis in females (Kishitake and Yamanouchi, 2003). Administration of parachlorophenilalanine (p-CPA), a compound that

selectively blocks serotonin synthesis, enhances most aspects of copulatory behavior in male rats (Malmnas and Meyerson, 1971; Salis and Dewsbury, 1971). In vertebrates, the main brain region containing serotonergic cell bodies is the raphe nucleus in the brainstem. The dorsal raphe nucleus (DRN) projects to many different brain areas, and some of them have gonadal steroid receptors and are involved in the control of reproduction (Fabre-Nys, 1998). The preoptic area (POA) is a region of the hypothalamus that plays an important role in the control of reproductive behavior in a wide variety of taxa including fish (Demski and Hornby, 1982; Goodson and Bass, 2001), and it receives serotonergic projections (Fabre-Nys, 1998). Drugs that increase serotonin activity or administration of 5-HT directly in the POA (Verma et al., 1989) decrease copulation rate in male rats and sexual receptivity and lordosis in females (Fabre-Nys, 1998). There is direct evidence that the DRN is involved in the inhibition of sexual behavior because lesions in this region increase lordosis in male rats (Kakeyama et al., 2002). Even if there is evidence of serotonergic projections to brain areas involved in reproductive behavior, the role of serotonin in modulating sexual behavior and reproductive function is not clear yet.

There are only a few studies showing evidence of sexual dimorphism in the serotonergic system. Levels of 5-HT, its precursor tryptophan, and its main catabolite 5-hydroxyindoleacetic acid (5-HIAA) in whole brains are higher in female rats than males, suggesting increased synthesis in females (Carlsson et al., 1985), and a study examining only the POA and the ventral medial hypothalamus also found higher 5-HT levels in female rats (Kakeyama et al., 2002). The mPOA in rats also shows a sexual dimorphism in the pattern of serotonergic fibers correlated with the morphological differences in the Sexually Dimorphic Nucleus (SDN) of the POA (Simerly et al., 1984).

Serotonin and social status

In various taxa including rodents (Blanchard et al., 1993), crustaceans (Edwards and Kravitz, 1997), fish (Winberg et al., 2001) and lizards (Larson and Summers, 2001), a link exists between serotonin activity, social status, and aggression level. In these studies, administering 5-HT agonist or SSRI reduces aggressive behavior or reverses the social status of the animal. Typically, in vertebrates high serotonergic activity is associated with submissive status and low serotonergic activity with high aggression and dominance status (Winberg et al., 1991; Winberg et al., 1997).

Serotonin and sex change

All these studies support a strong link between serotonin and the neuroendocrine mechanisms regulating both reproductive and aggressive behavior. Thus, serotonin is an excellent candidate molecule to act as a mediator between the social environment and the reproductive system in sex changing animals. Because increased levels of aggression are associated with the onset of sex change (Reavis and Grober, 1999), and in many species aggressive and dominant animals tend to have low serotonin levels, I predicted that high serotonin levels could be involved in the inhibition of sex change. Only two studies have considered the association between monoamines and sex change (Larson et al., 2003a; Larson et al., 2003b), and they provided some evidence that monoamines play a role in this process. Larson and colleagues (2003a) showed that the completion of gonadal sex change was inhibited in the saddleback wrasse *Thalassoma duperrey* by the SSRI sertraline and promoted by the antagonist ritanserin. However, they only examined gonadal histology and did not include any behavioral data, and therefore we do not know whether serotonin manipulations acted in the periphery only. Moreover, their sample size was

very small. Chapter 4 adds a strong behavioral component and addresses the problem within a social environment that is more similar to what fish experience in the wild.

In my work, I tested the hypothesis that *L. dalli* females, being subordinate, have elevated serotonergic activity, and this inhibits them from changing sex. Achieving dominant social status following male removal would reduce serotonin activity in the dominant female, and therefore remove the inhibitory action of 5-HT. A possible scenario is that the raphe nucleus sends inhibitory projections to the POA in females, and once released from this inhibition, the POA induces the expression of male sexual behavior and/or stimulates the hypothalamic-pituitary-gonadal (HPG) axis to start developing testes. Serotonin could inhibit sex change by altering arginine vasotocin (AVT) release from the POA because this peptide plays an important role in the control of sexual and aggressive behavior. There is evidence that 5-HT can affect the synthesis of AVT; in fact, males of the protogynous bluehead wrasses treated with the SSRI fluoxetine decrease AVT mRNA expression in the POA (Semsar et al., 2004). Also, fluoxetine can block the increase in aggressive behavior induced by arginine vasopressin (the AVT homologue in mammals) microinjections in the anterior hypothalamus of rats (Ferris et al., 1997). In *L. dalli,* males and sex changers have larger AVT immunoreactive neurons than females (Reavis and Grober, 1999).

Serotonin could affect steroid synthesis through the HPG axis but once the sex of the gonads begins to change, the shift in sex steroid production might also feed back on the serotonergic system and modulate its effects by affecting receptor densities. In fact, some studies suggest that POA levels of 5-HT or target tissue sensitivity to 5-HT might vary across the life of an individual and depend on the internal hormonal milieu (Hernández-Rauda and Aldegunde,

2002; Khan and Thomas, 1992), and estrogen can stimulate 5-HT_{1A} receptor-specific binding in the brain (Le Saux and Di Paolo, 2005).

Hermaphroditic Fish as Animal Models

Most of what is known about the neuroendocrinology of reproductive behavior and the link between serotonin and reproduction comes from rodent studies because of their easily quantifiable, predictable and stereotypic reproductive behavior. On the other hand, these strongly stereotypic characteristics make it harder to generalize the results to other species or investigate the subtle modulatory mechanisms that underlie more plastic reproductive behaviors. By using a sex changing species of fish as a model, we can follow in real time the events that result in the inhibition of female reproductive function and the induction of male gonads and sexual behavior in a naturally occurring context. Socially regulated sex change is an ideal model to address the crucial role of serotonin and steroid hormones in communicating changes in the social environment to the HPG axis. Because of its dual role in regulating aggression and reproduction, 5-HT is a reasonable candidate as a possible bridge between these systems in the regulation of sex change and as a key mediator between the external social environment and the internal neuroendocrinological environment. Also, given the well established social regulation of steroid hormones, it is an important next step to test changes in steroids levels in different tissues in response to social stimuli.

The Bluebanded Goby

The animal model that I used in my research is the bluebanded goby *Lythrypnus dalli.* It is a small benthic marine fish that inhabits rocky reefs along Southern California and Baja California

(Wiley, 1976). *L. dalli* can change sex in both directions (Reavis and Grober, 1999; Rodgers et al., 2007; St. Mary, 1994), but the population structure is typical of a protogynous species with males being both rarer and larger than females (Behrents, 1983; St. Mary, 1994). Even if both ovarian and testicular tissues are often found in the same individual, at the functional level, this species is a sequential hermaphrodite because individuals exhibit only one behavioral sex at a time (St. Mary, 1993, 1994).

L. dalli lives in social groups made of a male and several females and social status plays a critical role in regulating sexual transformations. *L. dalli* establishes a social hierarchy based mainly on body size through aggressive interactions (Behrents, 1983; Reavis and Grober, 1999), and males are usually dominant over females. When the male is removed, it is always the dominant female that undergoes sex change and becomes the new male (Reavis and Grober, 1999). Thus, it is straightforward to predict which female will be the eventual sex changer and record its behavior following male removal. While it is clear that the absence of a male induces sex change in the dominant female, it is not known which exactly are the sensory signals that trigger the onset of the process. Sex change in the protandrous direction follows similar social rules; in fact, if a male becomes subordinate to another male, it usually changes sex back into a female (Rodgers et al., 2007).

This species is particularly good to study the link between hormones, neurotransmitters and the reproductive system because the maturation and inhibition of male and female reproductive functions occur rapidly and the sex change process is completed in about two weeks (Reavis and Grober, 1999; Rodgers et al., 2007). In addition, this species is very small (20-40 mm standard length) so one can easily reconstitute a typical social group in a laboratory

aquarium, and it will present the suite of behavioral interactions similar to what is observed in the natural environment (Black et al., 2005b).

Dissertation Goals

In the first chapter of my dissertation, I examined the sensory cues that *L. dalli* females use to decide when the male is present, and therefore what are the sensory cues that inhibit sex change. I hypothesized that direct physical interactions exert a strong effect on the inhibition of protogynous sex change and that olfactory and visual stimuli alone are not sufficient to inhibit sex change in the dominant female. In the second chapter, I tested for the presence of diurnal rhythms in steroid hormones in *L. dalli* by collecting estradiol, T and KT at different times during the course of the day. This allowed us to assess what is the best time to sample hormones for behavioral studies. In the third chapter, I extracted and quantified hormones from brain, gonad and muscle to test whether they differ across tissues and how they relate to each other and to behavior. I also tested whether steroid levels differ between sexes and between females with different social status, and whether they change during the course of sex change. In the fourth chapter, I tested the role of serotonin in the inhibition of sex change by performing a variety of pharmacological manipulations of the serotonergic system combined with behavioral experiments. In the fifth chapter, I further studied the serotonergic system by visualizing, through an immunohistochemical analysis, the brain areas that synthesize serotonin. I tested whether there are differences in the area or number of serotonergic neurons between males, females and sex changing individuals.

The combined results of these studies add important insights into our understanding of the behavioral neuroendocrinology of sex change, the role of serotonin and sex steroids in the

modulation of social behavior, and the general mechanisms that translate social interactions into physiological consequences. They will also shed more light on the study of sex differences and, in particular, the neuroendocrine bases that underlie the presence of sexually dimorphic physiology and behavior.

CHAPTER 1

PREVENTING BEHAVIORAL INTERACTIONS WITH A MALE FACILITATES SEX CHANGE IN FEMALE BLUEBANDED GOBIES, *LYTHRYPNUS DALLI*

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Contributions:

R. L. Earley assisted in the editing of this manuscript and in the statistical analysis.

Abstract

Sex change in marine teleost fishes is commonly regulated by social factors. In species that exhibit protogynous sex change, such as the bluebanded goby, *Lythrypnus dalli*, the most dominant female typically initiates sex change when a male is removed from the social group. Females can use visual, chemical or tactile cues to assess the presence or absence of a male. The primary goal of our study was to determine whether the olfactory and visual presence of a male versus its behavioral interactions with females were important for mediating sex change. We exposed females to three different treatments: absence of a male, presence of a male that could physically interact with her, and presence of a male behind a barrier that allowed visual and olfactory interactions, but prohibited physical interactions. Sex change occurred in the absence of a male but not in the presence of a male that could physically interact with the female. The presence of a male behind the barrier did not prevent sex change but affected the timing of sex

change. Season appeared to have an effect on the latency to initiate male typical courtship, with a delay at the end of the reproductive season only when the male was present behind the barrier. We discuss the seasonal results in terms of *L. dalli* life history and the potential benefits and costs of changing sex late in the season in the presence or absence of aggressive reinforcement by the male. Our results identify direct behavioral interactions as an important proximate mechanism in the social regulation of sex change in *L. dalli*.

Introduction

Social interactions can exert potent effects on reproductive behavior, the distribution of matings within a social group, and ultimately fitness (Ellis, 1995). An extreme case of social regulation of reproduction is the inhibition of reproductive behavior and/or function in subordinates by dominant animals, a phenomenon that has been documented in a wide range of taxa from teleost fishes (Poeciliidae: Borowsky, 1987; Cichlidae: Francis et al., 1993) to mammals (dwarf mongooses: Creel et al., 1992). Subordinate individuals might perceive the dominant animal through visual, chemical or tactile cues, or a combination thereof, depending on species-specific differences in sensory systems. In honeybees, for example, chemical communication plays a major role in inhibiting workers from reproducing (Keller and Nonacs, 1993; Winston and Slessor, 1992) while in naked mole-rats tactile stimuli from the queen are important in preventing reproduction in subordinate females (Clarke and Faulkes, 2001).

Social regulation of reproductive behavior in sexually labile species often is manifested as control of the transition from one sex to the other by some aspect of the social environment (Fishelson, 1970; Robertson, 1972; Fricke and Fricke, 1977; Shapiro, 1979; Wright, 1988; Warner and Swearer, 1991; Warner et al., 1996; Baeza and Bauer, 2004). Although social

regulation of sex change is subtler than strict reproductive inhibition, the two phenomena are similar in that some individuals prevent others from achieving a particular reproductive phenotype, usually the phenotype with the greatest fitness potential.

In haremic protogynous fishes, females change sex to male, and males typically have the greatest reproductive success (St. Mary, 1994). Females do not change sex in the presence of a male, but his removal coupled with the presence of other females induces a female, usually the largest, to change sex (Fishelson, 1970; Robertson, 1972; Shapiro, 1981a; Reavis and Grober, 1999; for exceptions see Muñoz and Warner, 2003). Complete suppression of reproduction does not occur in these systems because both sexes are reproductively active, but some aspects of the male's presence inhibit females from developing male reproductive function and therefore from achieving the highest reproductive success. Although aggressive behavior by males has been previously suggested as a possible mechanism for preventing sex change in females of some territorial species (*Labroides dimidiatus*: Robertson, 1972; *Anthias squamipinnis*: Shapiro, 1981b*; Centropyge potteri*: Lutnesky, 1994), no studies to date have adequately partitioned the influence of male presence alone from behavioral interactions between the male and females in his harem.

We used the bluebanded goby (*Lythrypnus dalli*), a bidirectionally sex changing benthic marine fish to test explicitly whether preventing behavioral interactions with the male facilitates protogynous sex change in females. We use the general term behavioral interactions to account for the possibility that facilitation of sex change could be mediated by the removal of overt physical encounters or non-physical aggressive or courtship interactions. There is little known about group dynamics of *L. dalli* in the field. Behrents (1983) documented that this species lives at high densities but that individuals occupy social groups of 4-12 individuals within small

territories associated with a sea urchin. It is thus possible, given close proximity, that individuals could integrate olfactory, visual, or tactile (e.g., physical interaction, stimulation of lateral line system) cues to assess social group composition and individual status. Their small size allows us to establish semi-natural social groups in the laboratory, and their behavioral profile has been described in detail under both laboratory and field conditions (Reavis and Grober, 1999; Black et al., 2005b). Most importantly, the onset of sex change can be assessed behaviorally. Immediately after male removal the dominant female shows a peak in her rate of aggressive displacement and a nadir in submission. One to five days later she begins showing a distinct swimming behavior called jerking, which is typical of male courtship (Behrents, 1983; Reavis and Grober, 1999) and is associated with gonadal sex change. *L. dalli* establishes a social hierarchy based mainly on body size through aggressive interactions (Behrents, 1983; Reavis and Grober, 1999). Thus, it is straightforward to predict which female will be the eventual sex changer and record its behavior following male removal.

Previous studies on the inhibition or facilitation of sex change have focused primarily on disentangling the influence of olfactory and visual cues (Ross et al., 1983; Cole and Shapiro, 1995) and did not employ the appropriate controls to distinguish whether sex change inhibition was due to the mere presence of or behavioral interactions with the male. Some of these studies implicate tactile interactions with the male as an important cue regulating inhibition of sex change in females (Ross, 1981; Shapiro, 1983). The primary goal of our study was to begin to assess which cues might mediate protogynous sex change by disentangling the influence of male visual or olfactory presence from behavioral interactions between the male and dominant female. We acknowledge that behavioral interactions are inherently multimodal, being comprised of any combination of tactile, visual, and olfactory cues. Our aim is not to dissociate the various cues

that define behavioral interactions. Rather, we explore the relative influence of multimodal behavioral interactions versus visual/olfactory cues indicative only of male presence on the probability and dynamics of sex change. We manipulated the ability of a male to interact behaviorally with the females of a social group while either maintaining or eliminating the opportunity for visual and olfactory cues to be communicated between the sexes. Assessment of dominance status appears to be critical for regulating the decision to sex change in *L. dalli* (Rodgers et al., 2005). Because dominant status is most reliably assessed through behavioral cues, whether physical or not physical, we posited that removal of cues indicative of social subordination (i.e. male behavior) would be most salient in facilitating protogynous sex change in this species.

This experiment was conducted on fish collected during different seasons and given the potential for seasonal variation in reproductive behavior (Reavis and Grober, 1999), we accounted for season in all the analyses.

Materials and Methods

Study species

The bluebanded goby is a small benthic fish (20-45 mm adult standard length) that inhabits rocky reefs along southern California and Baja California, Mexico (Wiley, 1976). The majority of individuals reproduce during a single season (Behrents, 1983). Off the coast of Santa Catalina Island, California, where fish used in this study originate, *L. dalli* density ranges from 2 to 58 individuals/ m^2 depending on season and habitat complexity, with an average of about 24 individuals/m^2 (Behrents, 1983). The density peaks in the fall and is positively correlated with water temperature (Behrents, 1983). The bluebanded goby finds shelter from predators between

the spines of the sea urchin *Centrostephanus coronatus* and 90% of the fish are found associated with an urchin (Hartney and Grorud, 2002) with most commonly 1 to 4 fish per urchin. *L. dalli* establishes size-based dominance hierarchies with a dominant male that defends a nest in worm tubes or empty shells, and spawns with multiple females within a given season (Behrents, 1983). Individuals live in harem-structured groups, the composition and location of which are stable over time (Lorenzi et al., unpublished field data). The high abundance of predators around the urchins makes movements between adjacent groups very dangerous but no studies have examined interactions between groups in the wild. There is some evidence that individuals do emigrate from groups depending on shelter availability (Behrents, 1983; Steele, 2002) and that some males can migrate when a neighbouring male is artificially removed (Black et al., 2005b).

Females with developed eggs have been found in the wild from February to October with a peak from May to August (Wiley, 1976; Behrents, 1983). At the functional level this species is a sequential hermaphrodite because individuals exhibit only one behavioral sex (St. Mary, 1994; Reavis and Grober, 1999). The sexes can be distinguished using genital papilla shape (Wiley, 1976; St. Mary, 1993). Genital papilla length-to-width ratio is a good indicator of papilla shape and therefore of sex, with female ratio being close to 1 and male ratio ≥ 1.4 (Carlisle et al., 2000). Individuals with rounded, female-like papilla allocate more than 95% of their gonad to ovarian tissue, they lack accessory gonadal structures (AGS), and they do not spawn as a male. The AGS in this species consists of multiple long vesicles that produce a mucous secretion (Drilling and Grober, 2005). In other goby species, the secretion is used to make sperm trails that increase sperm viability in an aqueous medium (Marconato et al. 1996; Scaggiante et al., 1999). Male *L. dalli* typically have a thin pointy papilla, and always possess an AGS filled with mucous and sperm (Drilling and Grober, 2005). Males also defend a nest, provide parental care to the

eggs (Wiley, 1976; Behrents, 1983), and exhibit courtship behavior characterized by jerk swims, which are discontinuous, choppy movements usually from the nest to the female and back to the nest (Behrents, 1983; Reavis and Grober, 1999).

Experimental conditions

Fish were collected off the coast of Santa Catalina Island, California (CA Department of Fish and Game permit #: 803034-01), by SCUBA diving in September 2003 and March 2004 with the anaesthetic quinaldine (Sigma) and hand nets. Animals were collected with the same modalities and in the same site in both seasons. Because the experiments were conducted in two different seasons and because there is some evidence that season might affect the rate of sex change (Reavis and Grober, 1999), we accounted for it when comparing treatments (see "Data analysis" for details). Animals were shipped to Georgia State University, Atlanta (USA) where they initially were housed in communal tanks at a temperature of 19-20 ^{o}C, and a 12 :12 h light/dark photoperiod. The fish were fed brine shrimp twice daily with occasional supplements of Tetra-Min (Tetra; Blacksburg, PA, USA) marine flake food. Fish were removed from the communal tank and slightly anesthetized with tricaine methanesulfonate (MS222; Sigma). We recorded banding patterns for identification purposes, standard length (in millimetres), and wet body mass (in grams). The genital papilla was photographed, its length and width measured with calipers to calculate the papilla ratio (length/width), and its shape (rounded or pointed) was used for assignment of sex (Wiley, 1976; St. Mary, 1993). All fish that were smaller than 21 mm or that had ambiguous papilla shape were excluded from the study to avoid including immature individuals and fish in transitional states between the two sexes.

We established experimental groups consisting of a large male, a large female and 2 small females in 38 l aquaria with similar conditions as the communal tanks. Males were at least

3 mm larger than the largest female and the largest females were at least 4 mm longer than the small females in the same social group, which allowed us to identify unambiguously the dominant female and thus the predicted sex changer (Reavis and Grober, 1999). The presence of other females in the tank is important because sex change does not occur in social isolation (Carlisle et al., 2000). The standard length of each class of fish was similar across treatments (Table 1.1). A plastic screen divider (Lee's Aquarium & Pet Products, San Marcos, CA, USA) separated the tank into one small compartment (approximately one-third of the tank) and one large compartment. The divider was clear with holes of 1.5 mm diameter, and allowed visual and olfactory communication but no physical contact between fish in the different compartments. A test with a coloured plume (methylene blue) showed that there was circulation of water through the divider from the side housing the male to the side containing the females; there was no significant difference in the rate of movement of the plume in a tank with or without divider (t-test: $t_8 = 1.14$, $p = 0.29$; with divider: 6.4 ± 0.40 s; without: 5.2 ± 0.97 s). A small PVC tube was placed in the large compartment of each tank as a nest site; a transparent acetate sheet was inserted into the tube as a lining to facilitate removal of egg clutches.

Three different treatments were established, each consisting of 10 groups: 1) social group with male present (M); 2) male behind the clear, porous barrier (MB); 3) male removed - no male present (NM). Each group (male and females) was allowed to acclimate to the new tank for 6 days and to establish a social hierarchy. On the sixth day (day –1 from male removal) and for the following 22 days, we conducted 10-min behavioral observation sessions in the morning and afternoon of each day. We observed the predicted sex changer (largest female) in the MB and NM conditions, and the male and largest female in the M condition. For these focal fish, we recorded the number of displacements (an approach within 5 cm of another fish that results in the

approached fish moving away) performed toward all others occupying the same compartment, the number of displacements received from the other fish, and the number of jerk swims (courtship behavior) performed towards the nest or towards the other individuals.

On the seventh day (day 0) of the M treatment the male was briefly netted and returned to the same side as the females, so that the dominant female had visual, olfactory and physical interactions with the male. On day 0 of the MB treatment, the male was moved from the social group to the opposite side of the divider so that visual and olfactory cues indicative of male presence were still accessible to a female but behavioral interactions with the male were prevented. On day 0 of the NM treatment, the male was removed completely from the tank so that there was no interaction at all with the dominant female. If sex changers spawned, we removed the acetate sheet and checked for fertilized eggs.

The experiment ended on day 22 from male manipulation, and predicted sex changers in all treatments were euthanized with a lethal concentration of tricaine methanesulfonate (MS222; Sigma). We again photographed their genital papillae, measured the length and width of each papilla, and we extracted and photographed the gonads of the predicted sex changers to assess whether they had developed testes and AGS. We chose the day that the largest female performed the first jerk swim as an indicator of sex change initiation. We considered a dominant female to have changed sex if she exhibited male typical behavior (jerking and defending a nest) and, if at the end of the experiment she had a pointy genital papilla, typical testis morphology, and an AGS. The presence of fertilized eggs has been used in the past as a criterion of sex change in *L. dalli*, (Reavis and Grober, 1999) but we did not use this measure because the presence of eggs, and subsequent fertilization, is highly dependent on the ovarian status of the two subordinate females.

Data analysis

We used Fisher's exact test with Tukey's post-hoc test of proportions to assess treatment differences in the probability of female sex change. We used one-way analyses of variance (ANOVAs) to examine treatment differences in: (1) rates of aggressive behavior (displacements/min) throughout the entire 22 d period and during the first 4 d following male manipulation; (2) rates of courtship behavior (jerks/min) throughout the entire 22 d period and during the 3 d prior to and the day of the first spawn (eggs present in the tube), and (3) change of papilla ratios from the start to the end of the experiment. We used paired t-tests to determine whether papilla ratios of the focal animal within each treatment changed during the study. Because eight groups (belonging to the MB or NM treatment) were run in the fall (September and October 2003) and the rest in the spring (April and May 2004) we accounted for season in the analyses. Two-factor ANOVAs assessed treatment (MB or NM) and season (fall and spring) effects on the latency to initiate sex change (first jerk swim) and rates of displacement and jerking behavior; the M treatment was not included in these analyses because all trials were conducted in the spring. Planned linear contrasts examined differences among the levels of treatment and treatment x season interaction. The P-values for all non-independent post-hoc and planned comparisons were adjusted using the sequential Dunn-Sidak procedure to minimize compounding Type I error. All analyses were performed on SAS version 8.2 (SAS Institute, Inc.) using FREQ, GLM, or MIXED procedures. Data are reported as means ± 1 SE.

Results

Probability of sex change and associated morphological changes

None of the 10 dominant females in the male present (M) treatment changed sex; they did not exhibit male-typical courtship behavior, retained female-typical rounded genital papilla, and average papilla ratio at the end of the experiment was not significantly different from initial values (paired t-test: $t_9 = -1.27$, $p = 0.24$; initial: 1.01 ± 0.06, final: 0.92 ± 0.03). The dominant females also had distinct ovaries and did not develop an AGS.

All of the dominant females in the NM treatment changed sex; they exhibited jerking courtship behavior consistently throughout the experiment, occupied the nest, spawned and fertilized eggs. Their genital papillae transformed into a male-typical pointy shape, and at the end of the experiment their papilla ratio was significantly larger than at the start of the experiment (paired t-test: $t_9 = 7.38$, $p = 0.0001$; initial: 0.92 ± 0.03, final: 2.07 ± 0.14). All of the NM dominant females developed a testis and AGS.

Of the 10 dominant females in the MB treatment, 8 changed sex. These sex-changers jerked consistently throughout the experiment occupied the nest, and developed testis and AGS. Their papillae transformed into a male-typical pointy shape and at the end of the experiment their papilla ratio was significantly larger than at the start of the experiment (paired t-test: $t_7 = 8.67$, $p = 0.0001$; initial: 1.03 ± 0.06, final: 1.74 ± 0.04). Six of the sex changers spawned and fertilized eggs. The two fish that did not change sex jerked occasionally, but did not develop AGS, and at the end of the experiment had ovaries full of developed eggs and female-typical papilla morphology.

There were highly significant differences in the frequency of sex change among treatments (Fisher's Exact test: $p < 0.0001$, $N = 30$). Occurrence of sex change was significantly

greater in the NM (Tukey: $q = 8.20$, $p < 0.05$) and MB (Tukey: $q = 5.98$, $p < 0.05$) treatments than the M treatment. There was no significant difference between NM and MB in the probability of sex change (Tukey: $q = 2.22$, $p > 0.05$).

Rates of displacement and courtship

We compared overall rates of aggressive behavior (displacements/min over the 22 d experimental period) of sex-changed dominant females from the NM (1.43 ± 0.14; N = 10) and MB (1.07 ± 0.12; N = 8) conditions, and males (1.26 ± 0.11; N = 10) and dominant females (0.98 ± 0.09; N = 10) from the M condition. There were significant overall differences in displacement rates (ANOVA: $F_{3, 34} = 3.17$, $p = 0.04$), with NM sex-changers showing greater displacement rates than females in the M treatment (Tukey's; $p < 0.05$); no other comparisons were significant. Previous work on this species (Reavis and Grober 1999) indicated that sex-changers exhibit robust peaks in displacement rates immediately following male removal, thus we also examined rates of behavior during the initial stages of sex change. There were highly significant differences among treatments in displacement rates performed during the first 4 days (from day 0, male removal, to day 3) of the experiment (ANOVA: $F_{3, 34} = 8.10$, $p = 0.0003$; Fig. 1.1). NM dominant females had significantly higher displacement rates than all the other groups (Tukey's, $p < 0.05$), but MB sex changers, males and females in M controls did not differ significantly.

The overall rate of jerking (jerks/min) over the 22 d experimental period did not differ among treatments (ANOVA: $F_{2, 25} = 0.85$, $p = 0.44$). Sex-changers in the NM (0.77 ± 0.17, N = 10) and MB (0.54 ± 0.10, N = 8) groups, and males in the M condition (0.47 ± 0.52, N = 10) exhibited the same rates of courtship. Like displacements, jerking rates are not consistent over time in *L. dalli*. Peak jerking rates occur just before spawning as the sex changer or male courts the females (Reavis and Grober, 1999). There also was no significant difference among

treatments in jerking rates three days before and on the day of first spawning (ANOVA: $F_{2, 23}$ = 1.46, p = 0.25; Fig. 1.1).

Timing of sex change

For each fish in each treatment we calculated the difference between initial and final papilla ratio. In this analysis we include only the 8 sex changers for the MB treatment because we were interested in comparing the rate of sex change between NM and MB, and the difference between sex changers and dominant females in the M treatment. We excluded the remaining 2 MB dominant females because, although they might have been changing sex slower, they did not exhibit male typical traits at the end of the experiment. Overall there were significant differences among treatments in the degree to which the papilla ratio changed over the course of the experiment (ANOVA: $F_{2, 25}$ = 32.69, p = 0.0001). The change in papilla ratio was significantly greater in NM sex changers than in MB sex changers and dominant females in M (Tukey's: $p < 0.05$). The change in papilla ratio was significantly greater in MB sex changers than dominant females in M groups (Tukey's: $p < 0.05$).

Dominant females began jerking significantly earlier in the NM groups (day 2.6 ± 0.7, N = 10) than in the MB groups (day 6.2 ± 1.5, N = 10) (ANOVA: $F_{1, 16}$ = 4.43, p = 0.05) and the results were almost identical if we included only the 8 sex changers for MB. There was, however, a significant treatment x season interaction (ANOVA; $F_{1, 16}$ = 5.94, p = 0.03; Fig. 1.2). In spring (April and May) there was no significant difference between NM and MB treatments in the latency to begin jerking ($F_{1, 16}$ = 0.08, p = 0.79). Females in MB groups conducted during the fall (September and October), however, started jerking significantly later than females in fall NM groups ($F_{1, 16}$ = 8.06, p = 0.01) and females in the spring MB groups ($F_{1, 16}$ = 11.97, p = 0.003).

In the absence of males (NM groups), there was no significant seasonal effect ($F_{1, 16} = 0.1$, $p = 0.75$).

There was no effect of season on the rate of jerking by the sex changer in NM (N = 10) and MB (N = 8) groups (ANOVA: season $F_{1, 14} = 3.60$, $p = 0.08$; treatment x season $F_{1, 14} = 0.39$, $p = 0.54$; Fig. 1.2) but rates of displacement were higher in spring for sex changers from both groups (ANOVA: season $F_{1, 14} = 8.34$, $p = 0.01$; treatment x season $F_{1, 14} = 0.07$, $p = 0.80$; Fig. 1.2).

Discussion

The results of our experiment demonstrate that the removal of direct behavioral interactions between the male and dominant female facilitate protogynous sex change in *L. dalli*. Our results also demonstrate that, in the absence of behavioral interactions, visual and/or olfactory cues emitted by a male can modulate the timing of sex change. Furthermore, it is possible that seasonal rhythms, whether externally or internally derived, might influence the magnitude of a dominant female's response to visual and/or chemical cues indicative of male presence. We discuss the implications of this three-tiered explanation for the mediation of sex change in the context of the existing literature on the social regulation of sexual phenotypes in fishes and other vertebrate taxa.

In our study, no sex change occurred when the male was present and able to interact physically with the dominant female (M condition). Eighty per cent of the dominant females changed sex when a tactile barrier prevented direct behavioral interactions between the male and the female (MB condition). When the male was completely removed from the aquarium (NM), all dominant females changed sex. These findings are consistent with social regulation of sex

change in this species (St. Mary, 1994; Reavis and Grober, 1999) and support our hypothesis that direct behavioral interactions exert an important influence on the final decision of a dominant female to change sex. Our results also are consistent with those of Shapiro (1983) and Ross (1981) who demonstrated that females of *Anthias squamipinnis* and *Thalassoma duperrey*, respectively, changed sex when they were physically separated, but still received visual or chemical cues, from the focal male. These two studies, however, either neglected to consider behavioral interactions (Ross, 1981) or lacked control groups to assess whether the delay in the onset of sex change was due to the presence of the male behind a barrier (Shapiro, 1983). Also, there are few species in which the mechanisms that influence sex change have been investigated despite the diversity of teleost families in which it occurs. Thus, we cannot assume that the same mechanisms apply to each species. It is interesting to note that a serranid, such as *Anthias squamipinnis*, might share with gobies a proximate cue involved in the inhibition of sex change.

The barrier in our experiments prevented any direct behavioral interactions between the male and dominant female. We are aware that it is difficult to distinguish strictly tactile stimuli (e.g., overt physical interactions) and general behavioral interactions (e.g. courtship and aggression without physical contact) comprised of multimodal cues, as proximate mechanisms mediating sex change. Without invasive manipulations, however, it is virtually impossible to partition these cues.

Behavioral interactions inform a female about her dominance status in the harem and reproductive potential as a male. If the male is unable to effectively subordinate all females due, for instance, to increased group or territory size, a female could change sex even if olfactory and visual stimuli from the male are still present (Robertson,, 1972; Lutnesky, 1994; Perry and Grober 2003). Our results are in agreement with encounter rate models (Lutnesky, 1994), but

while those models could encompass any sensory modality and any level of interaction between nearby individuals, our study provides evidence that encounters involving direct behavioral interactions play an important role in inhibiting sex change. Visual and chemical cues emitted by the male were not sufficient to inhibit protogynous sex change, as demonstrated clearly by the results of the MB treatment where dominant females had visual and chemical contact with the male, but exhibited a significant propensity to change sex.

The ability of females to assess their social environment using means above and beyond the mere presence of the male might be beneficial. For various reasons, the female might gain more information through direct multimodal behavioral interactions than from simply sensing male presence through visual or olfactory means. First, visual and olfactory cues might inform the female about male presence, while interactions with the male, particularly agonistic interactions, might inform her of her status in the social hierarchy. Second, during sex reversal in *L. dalli*, behavior changes well before the gonads and secondary sexual characteristics. Behavioral interactions in the form of courtship might inform the female either that a male is present or that another female has initiated sex change in her social group. Thus, information gathered by attending to the behavior of another individual might be more important for a female than focusing on potentially equivocal visual or hormonal messages. Contrary to other species (e.g., *Thalassoma bifasciatum*), there is no sexual colour dimorphism in *L. dalli* thus females cannot rely on abrupt colour changes as an indication that another individual has adopted the male phenotype.

Despite the clear importance of the removal of multimodal behavioral interactions in facilitating sex change, visual and olfactory cues indicative of male presence affected the rate of sex change. Differences in behavior (latency to start jerking; displacements) and morphology

(papilla ratio) between MB and NM treatments indicate that females perceive the male behind the partition and integrate information from visual and olfactory stimuli. *L. dalli* dominant females typically show peak displacement rates immediately following male removal (Reavis and Grober, 1999), suggesting that the first few days are critical for a female to establish dominance over other females. If visual/olfactory cues associated with male presence were not important, we would predict MB females to increase displacement rates after male removal in a similar way as NM females. Rather, the aggression rate of MB females was similar to that of dominant females in the presence of a male. Similarly, if the effects of visual/olfactory cues were negligible, we would predict MB females to initiate sex change in parallel with NM females. MB females, however, showed delayed sex change initiation (latency to first jerk swim) compared with NM females. Lastly, at the end of the experiment, the papilla ratio of MB sex changers was less male-like than NM sex changers, indicating that MB females might have initiated sex change later or that it progressed at a slower rate. Thus, although direct behavioral interactions are important in regulating final outcome (i.e. sex change), visual and olfactory cues indicative of male presence are important for mediating behavioral changes and the timing of sex change.

Experiments on the induction of sex change in isolated or all-female groups have implicated visual and chemical signals as stimulatory cues for sex change. Ross (1981) demonstrated that the visual presence of a small female *T. duperrey* is sufficient to induce sex change in a larger female located behind a tactile barrier. Similarly, olfactory cues emitted from conspecific females increased the rate of sex change in solitary females of *Coryphopterus glaucofraenum* (Cole and Shapiro, 1995). The relative importance of visual and olfactory cues requires further investigation, and is likely to be a function of mating and/or social system (e.g.,

haremic vs. territorial; group stability), habitat use (pelagic vs. benthic), and density. Visual and olfactory cues might also play different roles in the induction versus inhibition of sex change.

Seasonal cues also might be important in mediating how visual/olfactory information from the male is integrated. In the spring, the early reproductive season for *L. dalli* (Wiley, 1976; Behrents, 1983), females began jerking at the same time in all conditions without physical interaction (NM, MB). When the male is either physically or behaviorally absent early in the season, the benefits of changing sex and reproducing as a male for an entire season might significantly outweigh the costs of reallocating reproductive investment; reproductive success in *L. dalli* increases by two fold or more when assuming the male reproductive function (St. Mary, 1994). In the fall, which marks the end of the breeding season, MB females tended to delay sex change and two of them did not change sex. *L. dalli* is a short-lived species that rarely reproduces for more than one season (Behrents, 1983) and in which females have a relatively long inter-clutch interval (up to 3 weeks; St Mary, 1994). Given these factors, allocating energy to testis development and spawning as a male for a short time in the fall might be more costly relative to spawning as a female. If after a certain period of time, the male fails to provide behavioral reinforcement (e.g. aggression, courtship), females might opt to change sex even if late in the season. In contrast, all NM females changed sex and began jerking rapidly in both fall and spring because in the absence of all cues related to male presence, the dominant females' options are either to forego spawning altogether or allocate energy to sex change. Because our study provides only preliminary evidence for seasonal effects, we encourage future work that investigates how season and visual/olfactory cues interact to affect the timing or expression of sex change and to understand which mechanisms might be involved.

Behavioral interaction as a mechanism affecting reproductive function is widespread among social species, and the effects of behavioral interactions range from inhibition of sex change to complete reproductive suppression. Dominant female alpine marmots (Hacklander et al., 2003) and naked mole-rat queens (Clarke and Faulkes, 2001) use aggression to inhibit subordinate female reproduction. Social insect workers also use aggression to suppress reproduction in other female workers (honey bees: Visscher and Dukas, 1995; wasp: Platt et al., 2004). The aggressive interactions might not be as important for non-territorial species or species in which the male cannot monopolize resources (e.g., females, territories, spawning sites). Future studies are necessary to assess whether proximate behavioral mechanisms of sex change inhibition can be generalized to other sequential hermaphroditic species with different social and mating systems. Our findings provide support for behavioral interactions as an important mechanism controlling sex change inhibition (Robertson, 1972; Fricke & Fricke, 1977). However, the delay of sex change that we observed in the presence of a male behind a barrier shows that individuals integrate multiple levels of visual and olfactory information, which is used to guide the expression of behavior that is most suitable to the dynamic social conditions.

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Table 1.1: Standard length ranges (mm) and averages (mean ± SE) for the animals composing the social groups.

Treatment	Male	Dominant female	Larger subordinate female	Smaller subordinate female
MB	32.55 - 41.30 (36.58 ± 0.92)	27.30 - 33.40 (30.11 ± 0.63)	22.60 - 26.90 (24.77 ± 0.46)	21.95 - 25.65 (23.74 ± 0.44)
NM	33.30 - 44.70 (36.07 ± 1.08)	27.30 - 33.70 (29.34 ± 0.59)	22.95 - 26.15 (24.42 ± 0.37)	22.40 - 25.50 (24.06 ± 0.37)
M	33.00 - 44.20 (36.82 ± 1.36)	26.75 - 32.70 (29.75 ± 0.64)	22.55 - 27.20 (24.72 ± 0.49)	21.50 - 27.10 (23.98 ± 0.54)

Animals were measured to the nearest 0.05 mm. MB = male present behind the tactile barrier; NM = no male present; M = male present.

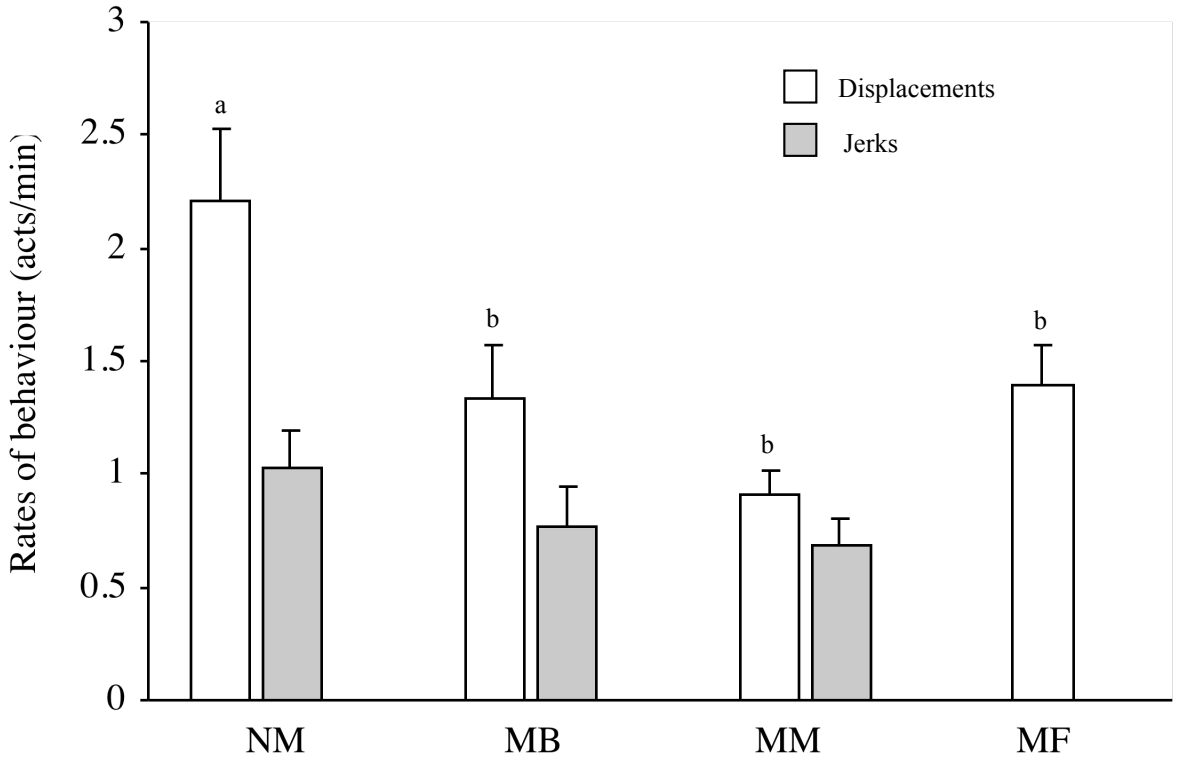

Figure 1.1: Comparison between rates of behaviour across treatments: the white bars represent displacement/min in the first four days from male removal; the grey bars represent jerks/minute in the day of spawning and 3 days before. The data refer to the sex changer for NM (N = 10) and MB (N = 8) groups, and to the males (MM) and dominant females (MF) for the M group (N = 10). Different letters represent statistical significant difference. Error bars represent the standard error of the mean.

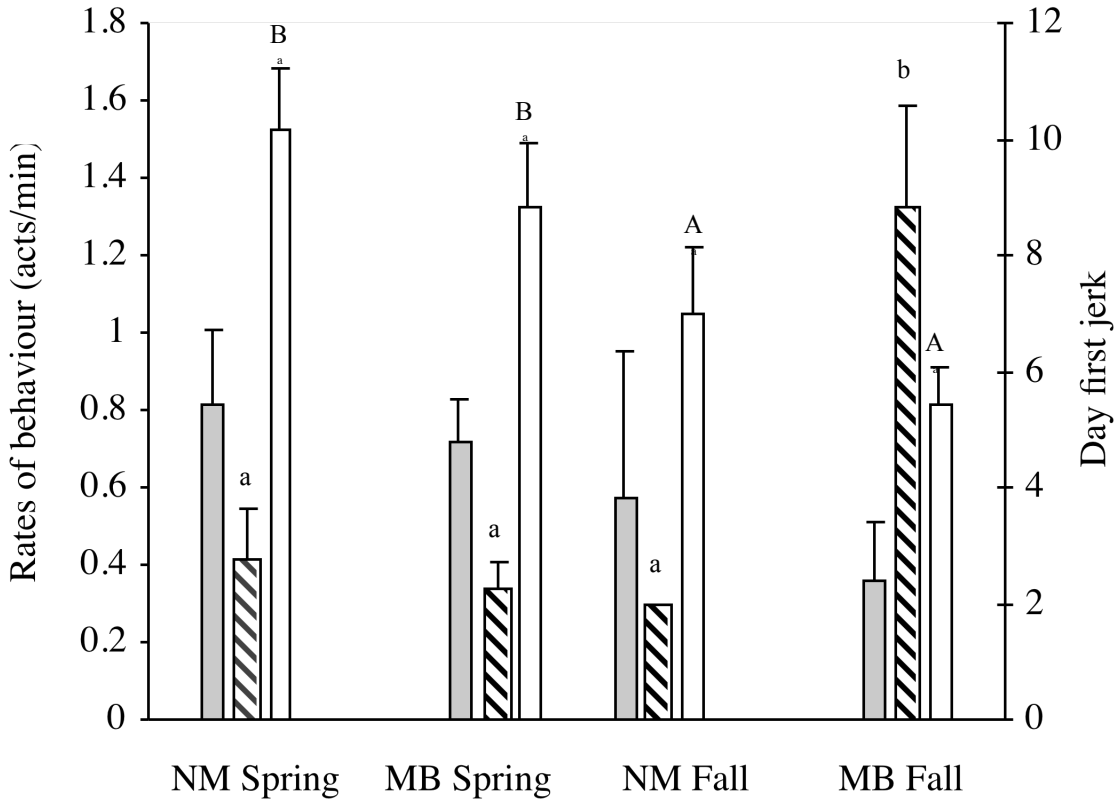

Figure 1.2: Effect of treatment by season interaction on different behaviours. The grey bars represent the sex changers rate of jerking over the whole experimental period, the white bars the overall rate of displacement, in the absence of the male (NM, spring N = 8; fall N = 2) or with the male behind the barrier (MB, spring N = 4, fall N = 4). The bars with diagonal pattern represent the first day after male removal that the dominant females started jerking in the absence of the male (NM, spring N = 8; fall N = 2) or with the male behind the barrier (MB, spring N = 4, fall N = 6). Different letters represent significant difference and error bars represent the standard error of the mean.

CHAPTER 2

DIURNAL PATTERNS AND SEX DIFFERENCES IN CORTISOL, 11-KETOTESTOSTERONE, TESTOSTERONE, AND 17B-ESTRADIOL IN THE BLUEBANDED GOBY (*LYTHRYPNUS DALLI*)

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Contributions:

R. L. Earley assisted in the hormone extraction and in the statistical analysis; E. W. Rodgers and D. R. Pepper assisted with collection of the water samples.

Abstract

The primary goals of this study were to evaluate diurnal patterns of and sex differences in the levels of cortisol, 11-ketotestosterone, testosterone, and 17β-estradiol in the sex changing bluebanded goby *Lythrypnus dalli.* Steroid hormones were collected from water samples and analyzed by enzyme immunoassay. During the breeding season, hormones were sampled from both males and females at seven time points between 0600 h and 2000 h. When comparing each time point separately, there were significant overall time effects for cortisol and 17β-estradiol. Cortisol concentrations were lowest at the 0800-1000 h sampling point and showed a qualitative peak in late morning (1000-1200 h). Concentrations of 17β-estradiol were elevated at the last sampling point (1800-2000 h). Broader temporal trends were revealed for testosterone and 11-

ketotestosterone concentrations, both of which were elevated in the morning. There were no sex differences in overall hormone concentrations or temporal profiles for cortisol, 11-ketotestosterone, or testosterone. Males and females showed similar diurnal patterns of 17β-estradiol but females had significantly higher water-borne 17β-estradiol levels than males. The results show the presence of diurnal changes in steroid hormone levels in male and female bluebanded gobies. The lack of sex differences in androgens suggests that males of this species, and perhaps other bidirectional sex changing species in which males do not exhibit prominent secondary sexual characteristics, do not require persistent elevations in 11-ketotestosterone or testosterone to maintain the male phenotype. Although the role of 17β-estradiol in maintaining sex differences in sexually plastic species is unclear, our results suggest that, of the hormones measured, 17β-estradiol has the greatest potential for future studies interested in this question.

Introduction

A central goal of research in behavioral endocrinology is to understand the relationships between circulating hormone levels and behavioral traits. Fluctuations in hormone levels in the same individual over time can be associated with a variety of non-behavioral factors including sex, season, and reproductive status (e.g., Goymann et al., 2004). Independent of these factors, hormones also follow endogenous diurnal rhythms in many vertebrates (Meier 1975). Therefore, determining the relationship between environmental or behavioral variation and individual differences in endocrine profiles requires an understanding of diurnal patterns in hormone secretion.

Diurnal rhythms of cortisol and corticosterone have received significant attention, in part because it is important to account for daily variation in these hormones when evaluating the

effects of stressors such as handling and sampling. In fishes, there is evidence that cortisol presents a diurnal rhythm characterized by higher values at night (Rance et al., 1982; Pickering and Pottinger, 1983; Nichols and Weisbart, 1984, Laidley and Leatherland, 1988), but the results during the day have shown tremendous variability. For example, no significant diurnal variation in cortisol exists in pre-spawning chum salmon (*Oncorhynchus keta*; Saito et al., 2004), while studies that have manipulated photoperiod length indicate that cortisol increases at the onset of the light phase in rainbow trout (*Oncorhynchus mykiss*; Reddy and Leatherland, 2003) and European sea bass (*Dicentrarchus labrax*; Cerda-Reverter et al., 1998). In male swordtails (*Xiphophorus helleri)*, corticoids are significantly higher at 0800 h than in the afternoon (Hannes and Franck, 1983). There also is clear evidence that daily cortisol peaks occur after feeding in rainbow trout (Bry, 1982) and brown trout (*Salmo trutta*; Pickering and Pottinger, 1983).

Variation in sex steroid levels, such as 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (KT) have been thoroughly investigated in fishes with regards to how circulating concentrations fluctuate with spawning cycle (Scott et al., 1984; Prat et al., 1990; Barnett and Pankhurst, 1994) but less in terms of diurnal rhythms. Most work on daily rhythms of sex steroids has been conducted in salmonids and other aquaculture species, while studies of this sort have been conducted less often in other fish models. As with cortisol, studies vary greatly in the demonstration of diurnal sex steroid rhythms. For instance, Bayarri and colleagues (2004) found a significant daily rhythm for T but not KT in male European sea bass; T levels peaked at 0700 h while levels of KT did not vary significantly during the day. In male cichlids (*Oreochromis mossambicus*) both androgens were higher at 0900 h than the rest of the day (Oliveira et al., 2001b). Conversely, water-borne KT and T in common dentex (*Dentex dentex*) were higher in the afternoon and early evening (Pavlidis et al., 2004), concordant with plasma T

results in male swordtails (Hannes and Franck, 1983). In the Japanese charr (*Salvelinus leucomaenis*) T showed a peak at 1800 h in both sexes and KT at 1500 h in males while E2 fluctuated without a significant pattern in females (Yamada et al., 2002). In the belted sandfish (*Serranus subligarius),* a simultaneous hermaphrodite, no daily rhythms were found for T or E2 (Cheek et al., 2000).

We explore daily variation in concentrations of the stress and sex hormones - cortisol, E2, T and KT - in the sex changing bluebanded goby, *Lythrypnus dalli*. The bluebanded goby is predominantly a protogynous fish (St. Mary, 1994; Reavis and Grober, 1999) but is capable of bidirectional sex change (St. Mary, 1994; Reavis and Grober, 1999; Rodgers et al., 2007*).* The well-established role of hormones in the process of sex change in this and other fishes (Carlisle et al., 2000; Frisch, 2004; Black et al., 2005a*),* suggests that understanding sexual and diurnal variation in hormone production is essential for generating a comprehensive picture of the behavioral endocrinology of sexually plastic fishes. The bluebanded goby is a social species living on rocky reefs from southern California to the west coast of Mexico (Wiley, 1976). The breeding season of the bluebanded goby ranges from May to September (Wiley, 1976). Hormones from both males and females were collected at intervals throughout the day during the breeding season using a non-invasive sampling technique. Our aims were to test the hypotheses that there are diurnal changes in endocrine profiles and sex differences in steroid hormone production.

Materials and Methods

Study organism: Collection and maintenance

Fish were caught off the coast of Santa Catalina Island, CA at 4.5-11.3 meters below the surface on the southeast and southwest points of Bird Rock using SCUBA (California Department of Fish & Game permit #: 803024-05 to VL). Briefly, groups of sea urchins (*Centrostephanus coronatus*) occupied by bluebanded gobies were located and the fish inhabiting the urchins were anesthetized with 10% quinaldine (30 ml quinaldine; 270 ml acetone), captured by hand netting, and transported in large plastic bottles back to the laboratory at the USC Wrigley Institute for Environmental Science. The fish were held in a large (60 x 94 cm^2) holding tank for three weeks until further processing; the holding tank was supplied continuously with seawater pumped directly from the cove adjacent to Bird Rock, and stocked with abundant shelter (8cm PVC tubes and kelp). The fish were fed either rehydrated brine shrimp or marine pellets twice daily between 0800-0900 h and 1700-1800 h. The research conducted herein was approved by the Georgia State University IACUC protocol No. A06004 (0708) and University of Southern California IACUC protocol No. 10262.

Sampling methods

All hormone samples were obtained on 7 July 2005, one day after the new moon; air temperature on this day ranged from 16.1 to 22.8 ºC (www.weather.com) and water temperatures (San Pedro buoy #46222; http://www.ndbc.noaa.gov/station_page.php?station=46222) ranged from 18.2 to 19.2 ºC over the course of the day. No artificial lights were operating in the laboratory and the fish were exposed only to the light penetrating large panel windows adjacent to the holding tank.

Eleven fish were removed from the holding tank with a net at each of seven time points (N=77 animals) from sunrise (0550 h) to sunset (2006 h): 0600, 0800, 1000, 1200, 1400, 1600,

and 1800 h (Pacific Standard Time). All fish that were used for a given time point were removed from the tank within 2 minutes; capture of males was especially rapid because they occupied the PVC shelters, and could be drawn into the net by tilting the shelter. Furthermore, it is important to note that all fish resumed normal perching (on the shelters or kelp) behavior and social interactions minutes after collection, which indicates that the procedure did not visibly disturb the fish for any length of time. We attempted to gather approximately equal numbers of males and females for each time point based on visual inspection of size; we chose not to closely inspect the genital papilla of each fish (using stereomicroscopy) prior to hormone sampling to minimize handling stress.

Individual fish were placed in 200 ml polypropylene beakers filled with 100 ml seawater (the same seawater as was pumped into the holding tank) at each time point for a 2 h hormone collection period; all beakers had been rinsed with ethanol and distilled water beforehand. The hormone collection beakers were placed in 2 cm of seawater to maintain temperatures approximating those in the holding tank and the beakers were spaced to eliminate visual contact between fish. Once the 2 h collection period was finished, the hormone collection water was poured through a net (rinsed with clean seawater) into a new, clean 200 ml polypropylene beaker, and fish were measured for standard length and their genital papillae were inspected to verify sex. The genital papilla is thin and pointy in males, and wide and rounded in females (Wiley, 1976). All animals used in this study had unambiguous genitalia; of the 77 individuals, N=32 males and N=45 females (see Figure 1 legend for sex distributions across time points). Mean standard length (mm) ± 1 SEM of females (F) and males (M) at each time point were: 0600-0800 (F: 24.03 ± 0.33; M: 30.09 ± 1.04), 0800-1000 (F: 26.24 ± 1.24; M: 33.18 ± 1.37), 1000-1200 (F: 25.29 ± 0.73; M: 31.2 ± 2.10), 1200-1400 (F: 25.44 ± 0.50; M: 32.35 ± 2.05),

1400-1600 (F: 26.73 ± 0.48; M: 34.87 ± 1.03), 1600-1800 (F: 25.68 ± 0.56; M: 33.85 ± 1.95), 1800-2000 (F: 26.65 ± 0.30; M: 31.79 ± 1.06). Following measurement, fish were placed in a new holding tank with the same dimensions as described above to prevent using the same fish multiple times.

Hormone was extracted from the water samples using C18 solid phase extraction columns (Lichrolut RP-18, 500 mg, 3.0 ml; Merck) fitted to a 12-port manifold (Earley et al., 2006; Rodgers et al., 2006). Columns first were primed using 2 consecutive washes with 2 ml HPLC grade methanol (MeOH) followed by 2 consecutive washes with 2 ml distilled water. Tubing was then fastened to the top of each column and was placed into the polypropylene beaker containing a water sample collected from the fish. The vacuum was engaged and the water sample was drawn up the tubing, passed through the column and the wastewater was collected in a bin inside the manifold. Salts were removed from the columns with 2 consecutive 2 ml washes of distilled water. The columns were then frozen and shipped to Georgia State University (GSU) for further processing. Freeze storage of water samples and columns has been determined not to impact steroid concentrations (Ellis et al., 2004). At GSU, the columns were thawed and purged with 2 consecutive 2 ml washes of distilled water. Hormone was eluted from the columns into 12 x 75 mm (6 ml) borosilicate vials by 2 consecutive 2 ml washes with HPLC grade MeOH. The 4 ml of eluted solvent was evaporated at 40 ºC (water bath) with a gentle stream of nitrogen (~ 0.7 bar), which was passed over the samples through an evaporating manifold. The resulting hormone pellet was resuspended in 840 µl of enzyme-immunoassay (EIA) buffer supplied with the kits (see below), and the samples were stored at -20 °C until assayed.

Cayman Chemicals Inc. enzyme immunoassay kits were used for all hormones (cortisol, KT, T, and E2). All samples were assayed in duplicate on two plates. All four hormones were assayed for each individual. Briefly, 50 µl of each sample was pipetted into the wells followed by 50 µl of acetylcholinesterase tracer and 50 µl of antiserum. Cortisol and KT plates were incubated overnight (18 h) on an orbital shaker at 4 ºC; T plates were incubated for 2 h on an orbital shaker at room temperature; E2 plates were incubated for 1 h on an orbital shaker at room temperature. The plates were then washed five times with wash buffer (provided with the kits), blotted dry, and 200 µl of Ellman's reagent was added to each well. The plate was wrapped in aluminum foil and placed on an orbital shaker for 60-120 min depending on the assay. Plates were read at 405 nm on a BioMek microplate reader. Two bluebanded goby pooled water extracts, which were derived from non-experimental fish (see next paragraph) and assayed in duplicate, were used as controls on each plate. Intra-assay coefficients of variation were (assay 1, assay 2): cortisol (6.58%, 1.24%), KT (2.67%, 10.3%), T (7.91%, 20.4%), and E2 (7.36%, 4.22%). Inter-assay coefficients of variation were: cortisol (6.78%), KT (5.38%), T (12.28%), and E2 (6.36%). All antisera are reported by Cayman Chemical Inc. to have 100% specificity for the focal steroid hormone. The cortisol antiserum cross-reacts with prednisolone (22%) and cortexolone (6.1%), cortisone (2%), and corticosterone (1.3%); all other cross-reactivity values were <0.2%. The KT antiserum shows no cross-reactivity with other androgens (e.g., testosterone <0.01%); see also Cuisset et al. (1994) for a report of low cross reactivities (<0.01%) of this antiserum with cortisol and cortisone. The T antiserum cross-reacts with 5α-dihydrotestosterone (27.4%), 5β-dihydrotestosterone (18.9%), androstenedione (3.7%), and 11-ketotestosterone (2.2%); all other cross reactivity values were <0.51%. The E2 antiserum cross-

reacts with estradiol-3-glucuronide (17%), and estrone (4%); all other cross-reactivity values were <0.57%.

The kits were validated for bluebanded goby water-extracted hormones by assessing parallelism of a serial dilution curve with the standard curve and quantitative recovery. Hormones were obtained from 48 non-experimental fish (males and females) using collection (8 h) and extraction methods similar to those described above. Evaporated samples were then resuspended in 60 µl 0.1 M phosphate buffer and combined into a pool of 2.9 ml. The pool was kept either at 1:1 (for serial dilutions) or diluted 1:16 in EIA buffer (for quantitative recovery), aliquoted and frozen.

Serial dilutions used 250 µl of the pooled, 'neat' (1:1) control. Briefly, 125 µl of this sample was transferred to a 1.5 ml microcentrifuge tube and mixed (by vortexing) with 125 µl of EIA buffer to create a 1:2 dilution; 125 µl of 1:2 dilution was mixed with an equal volume of EIA buffer to create a 1:4 dilution, and so on until 1:64 (cortisol, KT) or 1:128 (T, E2). The serial dilutions were assayed in duplicate using the EIA protocol described above. The log-logit transformed dilution curve was constructed using average % maximum binding and pg/ml concentrations for the eight dilution samples. The dilution curve was parallel to the standard curve for all hormones (comparison of slopes, Zar 1996, p. 355; cortisol: $t_{11} = 0.002$, $p = 0.99$; KT: $t_{11} = 0.001$, $p = 0.99$; T: $t_{12} = 0.089$, $p = 0.93$; E2: $t_{12} = 0.210$, $p = 0.84$).

A large (560 µl) sample of the bluebanded goby pooled control was used for quantitative recovery. One hundred microliters of this large sample was pipetted into a tube to constitute the 'neat' control, and 70 µl of the large sample was then pipetted into eight additional tubes and mixed with an equal volume of the standards provided with each enzyme immunoassay kit. Expected recovery concentrations were based on the known amount of cortisol, KT, T, or E2 in

the bluebanded goby control sample. Minimum observed recovery for cortisol, KT, T, and E2 was 93.3%, 92.6%, 62.3%, and 76.5% respectively. The slopes of the observed vs. expected curves were 0.995 (cortisol), 1.029 (KT), 0.815 (T) and 0.762 (E2), suggesting linear relationships between expected and observed values for all hormones. The sensitivities of the assays (plate 1, plate 2) were as follows: cortisol (15.20, 19.18 pg/ml); KT (1.37, 1.06 pg/ml); T (8.53, 8.17 pg/ml); E2 (11.30, 18.69 pg/ml). All data are presented as pg/sample (pg/ml multiplied by 0.84 ml, which was the amount of EIA buffer used to reconstitute the sample).

Statistical analyses

Two-factor analysis of covariance (ANCOVA) with standard length as a covariate was used to assess differences in hormone concentrations (pg/sample) among the seven time points and between the sexes; the use of different animals at each sampling period precluded within-subjects analyses. Interactions between the main effects (time and sex) and standard length were not significant for any of the hormones and thus were eliminated to produce a reduced model (time x standard length; cortisol: $F_{6, 54} = 0.97$, $P = 0.45$, KT: $F_{6, 54} = 0.63$, $P = 0.71$, T: $F_{6, 55} = 0.38$, $P = 0.89$, E2: $F_{6, 55} = 0.38$, $P = 0.89$; sex x standard length; cortisol: $F_{1, 54} = 0.31$, $P = 0.58$, KT: $F_{1, 54} = 1.39$, $P = 0.25$, T: $F_{1, 55} = 0.09$, $P = 0.76$, E2: $F_{1, 55} = 0.07$, $P = 0.78$). Variance in standard length was homogeneous among time points (Brown-Forsythe test: $F_{6, 70} = 1.43$, $P = 0.21$). T was natural-log transformed, E2 was $y^{0.25}$ transformed, cortisol was square root transformed, and KT was inverse transformed to achieve normality (Shapiro-Wilk goodness of fit, $P > 0.13$). Linear contrasts were used to investigate broader differences in the time course of hormone release (e.g., morning versus afternoon).

Results

Comparing all seven sampling points, there were significant overall time effects only for cortisol and E2 (Table 2.1). Cortisol concentrations showed a significant nadir at the 0800-1000 h sampling point (Student's t-test: $P < 0.05$) and a qualitative peak in late morning (1000-1200 h)(Fig. 2.1.A). E2 concentrations were significantly elevated at the last sampling point (1800-2000 h) relative to all others (Student's t-test: $P < 0.05$), except the one that immediately preceded it (1600-1800 h); E2 concentrations at the 1600-1800 h sampling point were significantly greater than at 0600-0800 h and 1000-1200 h (Student's t-test: $P < 0.05$)(Fig. 2.1.D).

Broader analyses of differences in the time course of hormone release indicated that cortisol concentrations were significantly lower in early morning (0600-1000 h) than in late morning, afternoon, and evening combined (1000-2000 h)(linear contrast: $F_{1, 59} = 6.62$, $P = 0.013$). KT concentrations were significantly higher at the 0600-0800 h time point (Fig. 2.1.B) than all other time points combined (linear contrast: $F_{1, 59} = 5.83$, $P = 0.02$). T concentrations were significantly higher in the morning (0600-1200 h)(Fig. 2.1.C) than in the afternoon and evening combined (1200-2000 h)(linear contrast: $F_{1, 60} = 4.81$, $P = 0.03$). Lastly, E2 concentrations were significantly higher in the late afternoon (1600-2000 h) than in all other time points combined (linear contrast: $F_{1, 62} = 21.41$, $P < 0.0001$).

There were no sex differences in overall hormone concentrations or temporal profiles (time x sex) for cortisol, KT, or T (Table 2.1). There was, however, a pronounced sex effect for E2 (Table 2.1) with females having significantly higher overall water-borne E2 levels than males; males and females showed similar diurnal patterns of estradiol (time x sex interaction not

significant). Hormone concentrations did not covary significantly with standard length (Table 2.1).

Discussion

Our results demonstrate prominent diurnal patterns of water-borne E2 and cortisol secretion in the sexually plastic bluebanded goby (*Lythrypnus dalli),* and broader daily variations in T and KT. Although our data suggest that these steroid hormones follow a discernable temporal pattern, we sampled hormones over only a 14 h period between 0600 h and 2000 h. To reveal a true diurnal cycle would require at least two days of continuous hormone sampling and the demonstration that similar peaks and troughs in steroid hormone concentrations occur across days.

Cortisol concentrations were lowest at the 0800-1000 h sampling point, exhibited a qualitative peak in late morning (1000-1200 h) and intermediate values the rest of the day. We fed our fish between 0800 and 0900 h suggesting that the observed increase in cortisol between the 0800-1000 h and 1000-1200 h time periods could represent a post-feeding cortisol response, which is typical in other fish species (Bry, 1982; Pickering and Pottinger, 1983). We also observed low cortisol concentrations before the feeding peak, which corroborates results of a study in immature rainbow trout (Laidley and Leatherland, 1988); the exact time of the cortisol trough in immature rainbow trout ranged between 0800 and 1200 h depending on feeding time and season. It is interesting to note, however, that we did not see a peak in cortisol concentrations following the afternoon feeding period (1700-1800 h), suggesting at least three alternative explanations: (1) cortisol increases are not tightly coupled with feeding *per se*, (2) there may be differential cortisol responses to feeding in the morning and afternoon due to the time interval

between feeding bouts (e.g., 8-10 h between morning and afternoon; 14-16 h between afternoon and next morning), or (3) afternoon feeding-induced peaks of cortisol are masked by diurnal peaks during this time period (i.e., there is a confound between feeding time and endogenous diurnal patterns).

One study in the European sea bass showed that cortisol levels peak with the onset of the light phase, decline in early afternoon, and increase again in the evening (Cerda-Reverter et al., 1998). In juvenile rainbow trout cortisol levels were very low in early morning just before the light phase, peaked in late morning, and dropped in the afternoon (Reddy and Leatherland, 2003). These results are somewhat similar to the pattern we observed in the bluebanded gobies with respect to an early morning trough and an increase in late morning. The decline in cortisol that we observed, however, occurred after the onset of light (0550 h). In European sea bass and juvenile rainbow trout cortisol decreased or peaked again in the afternoon, respectively, while in bluebanded gobies cortisol concentrations remained quite constant and moderate in the afternoon.

There also is evidence for seasonal effects on diurnal patterns of cortisol secretion. During the summer, cortisol levels of immature rainbow trout peaked at midnight and between 0700 and 0900 h. The same species tested in the winter did not present any diurnal pattern in cortisol levels (Rance et al., 1982). In the future, it will be interesting to ascertain whether diurnal patterns of cortisol in bluebanded gobies are contingent upon season and associated features such as reproductive versus non-reproductive periods or differences in water temperature.

Regarding diurnal patterns of sex steroid secretion in bluebanded gobies, E2 levels were elevated in the late evening while androgens (T, KT) tended to be higher in the morning. These

findings parallel those in male swordtails, cichlids, and European sea bass where androgens are high in the morning and decrease in the afternoon (respectively: Hannes and Franck, 1983, Oliveira et al., 2001b; Bayarri et al., 2004). Our results for E2 differ from those found in the belted sandfish, a simultaneous hermaphrodite, for which no daily E2 rhythms were uncovered (Cheek et al., 2000). Our results are, however, very similar to those found in *Pseudolabrus sieboldi* (Sundaray et al., 2003), which like the bluebanded goby, is predominantly a protogynous sex changing fish. Sundaray and colleagues (2003) demonstrated in male *P. sieboldi* that T and KT levels were higher in the morning until 0900 h, and declined at the next sampling point of 1200 h. They also found that E2 levels were very low except for a peak at 1500 h. Despite that study examining only males, it is consistent with our results that show higher androgens levels in the morning and a peak in E2 in the afternoon in both sexes. A previous study on bluebanded gobies found higher KT values in the morning but these were not significantly different from afternoon samples in newly sex-changed individuals (Black et al., 2005b). In light of our data on daily hormone patterns, the data from Black et al. (2005b) is not surprising because their morning sampling occurred after 0900 h when KT concentrations should already have dropped markedly. In the same study Black et al. (2005b) found that morning, but not total, KT levels correlated with aggressive behavior. Oliveira et al.'s (2001b) work on the relationship between social interactions and androgen levels also testifies to the importance of controlling for time of day when studying hormone-behavior relationships. In that study, androgen levels in control animals declined as the day progressed, making it essential to account for time of day in fish that were exposed to a social challenge and sampled up to 6 h following the interaction.

The significant peak of E2 in bluebanded gobies after 1800 h might indicate ovulation and precede spawning. Bluebanded gobies spawn in captivity at different times of the day (personal observation, VL) but likely in late evening or early morning; most spawnings occur outside the daily behavioral observations (0800h to 1800h), as evidenced by the appearance of eggs the next morning. Indeed, female *P. sieboldi* show E2 peaks at 0300 h and this corresponds with vitellogenic follicle activity (Ohta et al., 2001). Both E2 and T peak during vitellogenesis in the protandrous Asian seabass (*Lates calcarifer;* Guiguen et al., 1993), and in the spiny damselfish (*Acanthochromis polyacanthus*), females have the highest E2 values when oocytes are at the final stage of maturation or ovulated (Pankhurst et al., 1999). Also, E2 levels correlate with an increase in vitellogenic oocytes in the bi-directional sex changing broad-barred goby (*Gobiodon histrio*; Kroon et al., 2003). Although the spawning status of females in our study was unknown, it is possible that the late afternoon peak in E2 represents spawning anticipation. We noted substantial variation in untransformed E2 levels at this time point (Figure 1). Perhaps this is the result of sampling females with oocytes at different stages of development, a hypothesis that will require confirmation in future experiments.

In our study we found a large peak in E2 in the afternoon and we would expect also a corresponding peak in T because E2 released by fish ovaries typically is synthesized via aromatization of T (e.g., Lee et al., 2006). We did not observe an afternoon increase in T so this might indicate that E2 is following a different synthetic pathway. In fact, in the protogynous *P. sieboldi*, E2 is produced from estrone instead of T (Ohta et al., 2001). This alternative pathway also could operate in bluebanded gobies*,* perhaps in tandem with aromatization of T; female bluebanded gobies do show higher aromatase activity than males (Black et al., 2005a). The possibility that bluebanded gobies uses divergent pathways for E2 synthesis deserves further

investigation because it could represent a shared mechanism for E2 production in hermaphroditic species, and because it has implications for understanding seemingly discordant diurnal patterns of biosynthetically related steroid hormones.

When examining sex differences in hormone levels, we revealed that there was a sex difference only in E2. We did not find any differences in cortisol levels between males and females, which corroborates the lack of sex differences in cortisol levels in the protandrous anemonefish (*Amphiprion melanopus*; Godwin and Thomas, 1993). In bluebanded gobies, as in many other sex-changing species (Nakamura et al., 1989; Guiguen et al., 1993; Kroon and Liley, 2000; Lone et al., 2001; Bhandari et al., 2003; Kroon et al., 2003), females have higher E2 levels than males. Studies using exogenous administration of E2 and/or aromatase inhibitors have indeed shown that E2 plays an important role in the regulation of sex change (for a review see Frisch, 2004).

KT has been considered the primary male androgen in fishes and typically is higher in males than females (reviewed by Borg, 1994). This also is true for some protogynous sex changing species such as the blackeye goby (*Coryphopterus nicholsii*; Kroon and Liley, 2000) and the honeycomb grouper *(Epinephelus merra;* Bhandari et al., 2003), and some protandrous species such as the sobaity (*Sparidentex hasta*; Lone et al., 2001), the seabass (Guiguen et al., 1993), and the anemonefish (Godwin and Thomas, 1993). Interestingly, bluebanded goby females and males have comparable KT regardless of the time point at which sampling occurred. This result parallels those found in the bi-directional sex changing broad-barred goby (Kroon et al., 2003), where KT levels were very low or undetectable in all sexual stages, and in the protogynous saddleback wrasse (*Thalassoma duperrey*) where circulating levels of KT were not significantly different between females and males (Nakamura et al., 1989). On the other hand,

Nakamura and colleagues (1989) found that even if there were no sex differences in circulating hormones, the testis show a greater capacity for in vitro KT production than the ovary so perhaps tissue specific differences play a major role. Our results confirm those of Rodgers et al. (2006) who found no sex difference in bluebanded goby KT levels unless the males were sampled while they were parenting a clutch of eggs. KT in bluebanded gobies is known to affect genital papilla masculinization (Carlisle et al., 2000) and correlate with percent testicular tissue and the size of the accessory gonadal structure (Black et al., 2005b) indicating a putative role for KT in supporting the initial development of the male phenotype during protogynous sex change. The importance of KT in maintaining the male phenotype, however, might be negligible given the lack of sex differences observed in these studies. Persistent elevations in KT, an androgen known to potentiate the development of exaggerated male phenotypic traits (Oliveira et al., 2001a; Mayer et al., 2004) may be unnecessary in bluebanded gobies and other bi-directional sex changers because of the absence of dramatic male secondary sexual characteristics. It also is possible that KT serves more general purposes such as tissue growth and development (Thorarensen et al., 1996) that are sex-independent, thereby precluding dramatic sex differences in hormone concentrations (Schultz et al., 2005). It also is important to note that bluebanded goby females can often have small pockets of testicular tissue present in the gonad (St. Mary, 1993) even while they are reproducing as female and have vitellogenic oocytes. Because KT is associated with spermatogenesis, this might explain why female KT levels are comparable to those found in males.

The lack of sex difference in T levels is not surprising because in fishes T is present in both males and female, and females often have higher levels (Borg, 1994) especially during the spawning season because T is used as a precursor for E2 during aromatization (see above for

alternative mechanisms). For example, no sex differences in T were found in the protandrous sobaity (Lone et al., 2001), in the protogynous saddleback wrasse (Nakamura et al., 1989) and honeycomb grouper (*E. merra*; Bhandari et al., 2003), or in the bi-directional broad-barred goby (Kroon et al., 2003). It should be noted, however, that there are also cases in fish where males have higher T than females (blackeye goby, Kroon and Liley, 2000; common dentex, Pavlidis et al., 2000).

We did not find any evidence of a sex difference in diurnal hormone patterns (time x sex interaction). In gulf killifish (*Fundulus grandis*) there is some evidence of a sex difference in diurnal pattern with males having two peaks in cortisol (at 0600 h and 1400 h) and females having only a peak at 0600 h (Garcia and Meier, 1973). Nevertheless, most other studies on diurnal patterns examine either males or females.

In the present study, we used a technique that entailed extracting steroid hormones from the water. This technique has the advantages of reducing handling stress and allowing for non-sacrificial hormone sampling in small fishes. Because steroid hormones released through the gills, feces and urine of fishes can be sampled reliably from the water, and because water-borne steroid concentrations appear to be indicative of circulating levels, this non-invasive sampling technique has become increasingly popular for measuring stress and sex hormones, including all of those measured in this study (Scott and Sorensen, 1994, Greenwood et al., 2001; Scott et al., 2001; Hirschenhauser et al., 2004; Ellis et al., 2004; Pavlidis et al., 2004; Black et al., 2005b; Seibre et al., 2007). As we discussed above, our results are consistent with previous studies in the bluebanded goby and with studies in other species where hormones were measured from blood giving further support to this valuable technique.

In conclusion, we have demonstrated robust diurnal patterns of sex and stress hormones in a sexually plastic goby using a non-invasive sampling technique. Cortisol and estradiol showed marked changes in the morning and evening, respectively, while both androgens showed gradual changes detectable only with broader scale analyses. These results suggest that steroid hormones, even those that are intricately linked (e.g., T and E2) follow independent diurnal trajectories, thereby necessitating standalone diurnal assays for each steroid. Our data correspond well with those of other fish species but we still are unable to make comparative generalizations about diurnal hormone patterns owing to tremendous variability across studies in the techniques, contexts, and times of day at which hormones are sampled. It is nonetheless essential that we understand diurnal changes in hormone levels, and properly account for time of day in studies of hormone-behavior relationships. We confirmed previous reports (Rodgers et al., 2006) that KT concentrations do not differ between male and female bluebanded gobies. This result contrasts a body of literature implicating KT as the primary male hormone in fishes (but see Schultz et al., 2005 and references therein). Some unique features of the bluebanded goby – notably sexual plasticity and lack of dramatic secondary sexual characteristics in males - may preclude sex differences in KT. Lastly, E2 concentrations were strongly sexually dimorphic, with females consistently (throughout the day) having higher levels than males. This suggests that, of the hormones measured in this study, E2 has the greatest potential for being involved in the maintenance of divergent phenotypes in a bidirectional sex changing species.

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Table 2.1: The effects of time, sex, and standard length on hormone concentrations derived from a reduced ANCOVA model.

Hormone	Effect	F-value	P-value	Means
Cortisol	Time	$F_{6, 61} = 2.76$	0.019	
	Sex	$F_{1, 61} = 0.13$	0.720	female: 251.01 ± 32.67, male: 246.13 ± 41.58
	Time x Sex	$F_{6, 61} = 0.46$	0.833	
	Standard Length	$F_{1, 61} = 0.73$	0.397	
11-Ketotestosterone	Time	$F_{6, 62} = 0.39$	0.881	
	Sex	$F_{1, 62} = 0.55$	0.459	female: 19.07 ± 2.96, male: 18.44 ± 1.92
	Time x Sex	$F_{6, 62} = 0.44$	0.846	
	Standard Length	$F_{1, 62} = 1.71$	0.196	
Testosterone	Time	$F_{6, 62} = 1.21$	0.311	
	Sex	$F_{1, 62} = 1.70$	0.197	female: 156.71 ± 12.62, male: 116.44 ± 9.27
	Time x Sex	$F_{6, 62} = 1.02$	0.421	
	Standard Length	$F_{1, 62} = 0.04$	0.842	
17β-Estradiol	Time	$F_{6, 62} = 4.23$	0.001	
	Sex	$F_{1, 62} = 9.88$	0.003	female: 332.7 ± 86.69, male: 66.53 ± 8.13
	Time x Sex	$F_{6, 62} = 1.78$	0.119	
	Standard Length	$F_{1, 62} = 0.01$	0.925	

Mean (pg/sample) ± SEM hormone concentrations are displayed for the sex effect (N=32 males; N=45 females in all cases).

A.

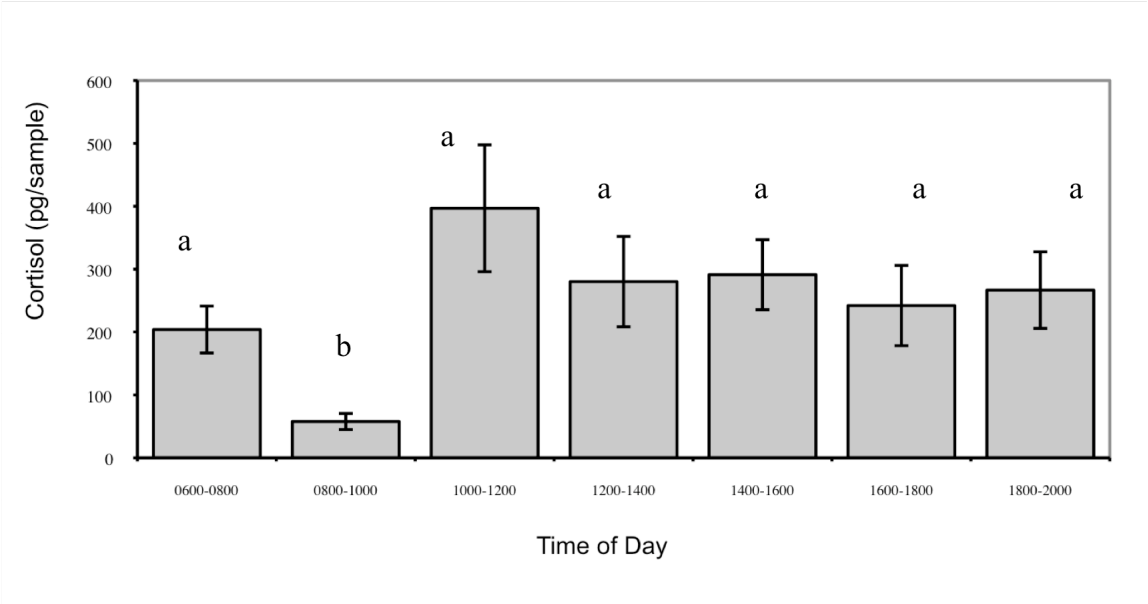
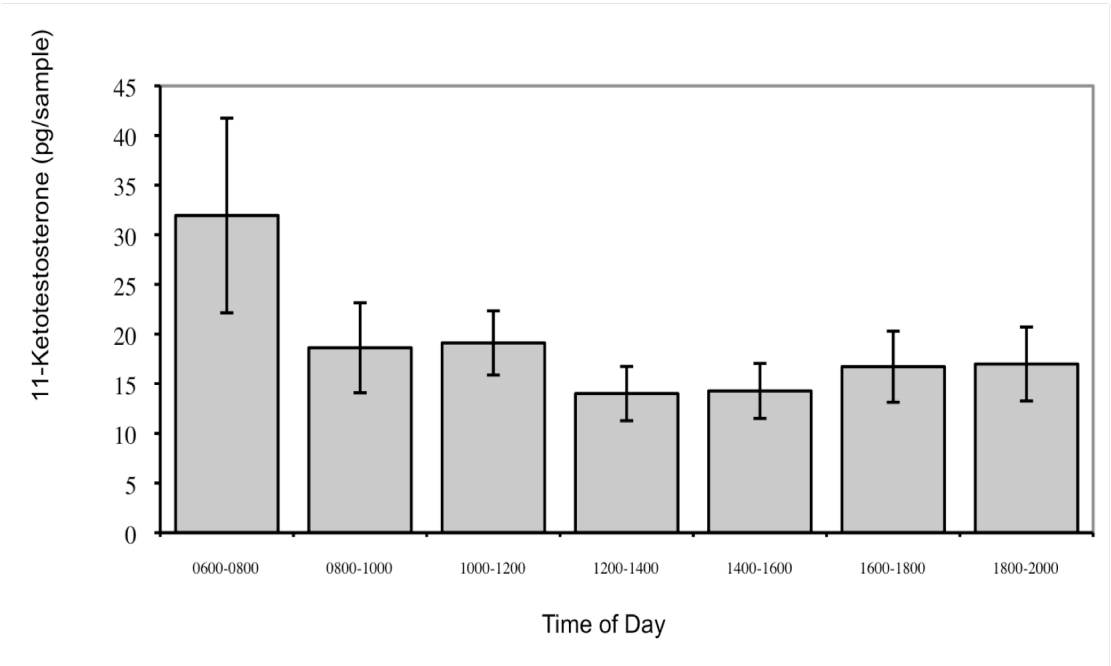


Figure 2.1: Time course of water-borne hormone concentrations (pg/sample) for (A) cortisol, (B) 11-ketotestosterone, (C) testosterone, and (D) 17β-estradiol. Water-borne hormone concentrations were pooled for males and females because there was no sex difference in temporal profile (Table 1; time x sex). Despite all hormone values being transformed for analysis, the histogram bars depict untransformed means ± 1 SEM. N=11 for each bar in each graph. The sex distribution is as follows, with F=female, M=male: 0600-0800 (3F, 8M), 0800-1000 (7F, 4M), 1000-1200 (9F, 2M), 1200-1400 (9F, 2M), 1400-1600 (8F, 3M), 1600-1800 (5F, 6M), 1800-2000 (4F, 7M). For cortisol and E2, histogram bars with different letters are significantly different (Student's t-test; $P < 0.05$); for KT and T, see Results section for comparisons across broader time scales.

Figure 2.1: Continued

B.



C.

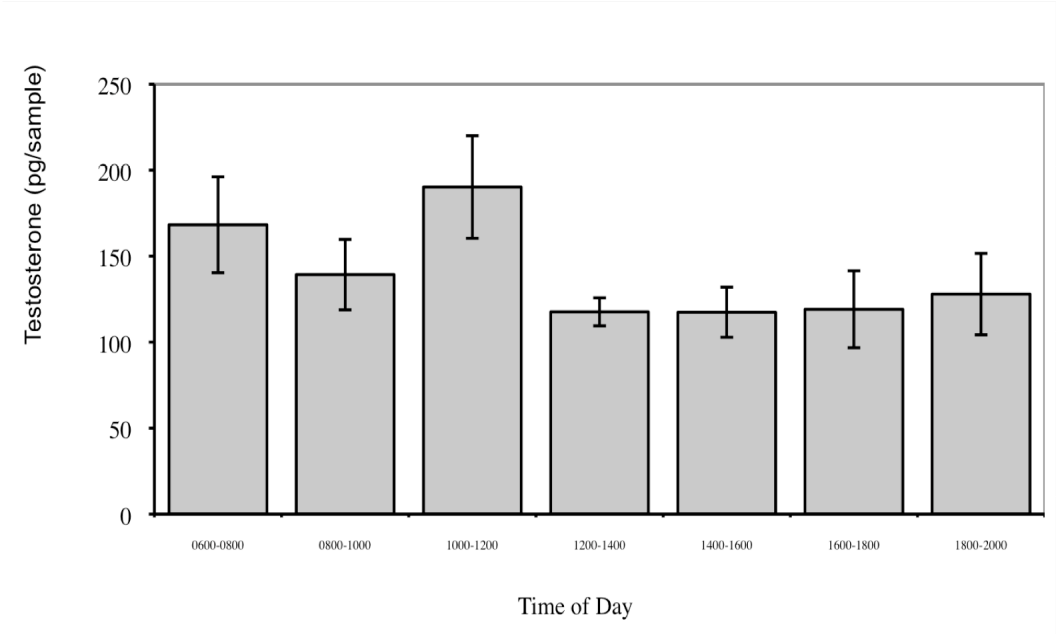
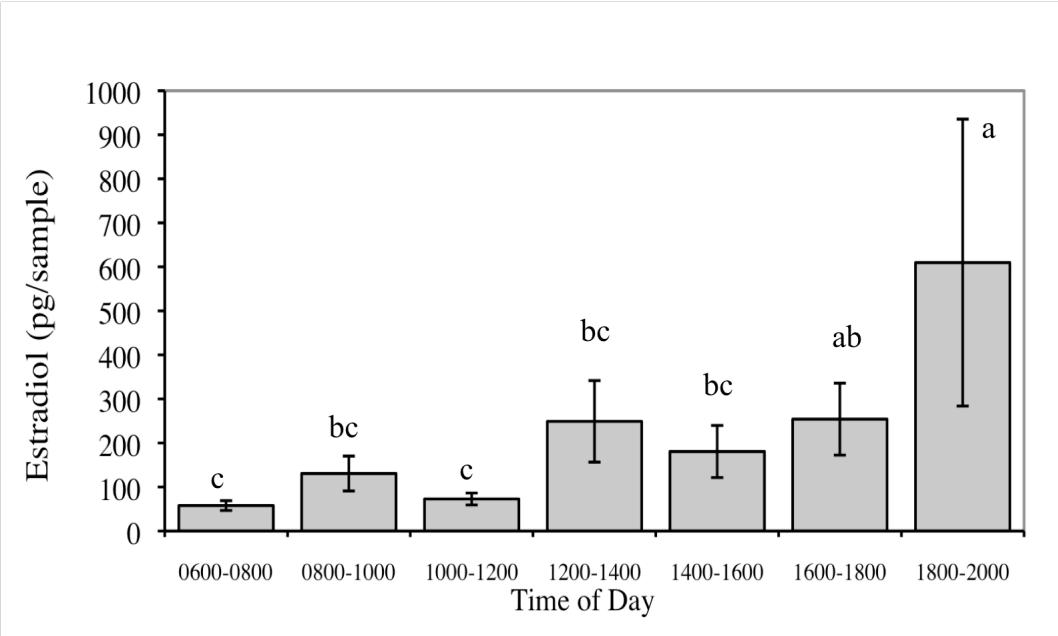


Figure 2.1: Continued

D.



CHAPTER 3

SEX CHANGE, SOCIAL STATUS AND STEROIDS: DIFFERENT TISSUES HAVE DIFFERENT PERSPECTIVES

Abstract

Sex steroids can both modulate and be modulated by behavior, and their actions are regulated by complex interactions between multiple sources and their targets. While gonadal steroids delivered via circulation can affect behavior, changes in local steroid synthesis can also be important. The bluebanded goby (*Lythrypnus dalli*) is a sexually plastic fish for which dominance interactions are the key to determining sexual phenotype, and sex steroids can play a role in modulating both aggression and reproduction. We examined changes in steroid levels among several tissues during socially induced sex change. We also tested for differences among females of different social status. We collected brain, gonads and body muscle at either 24 hours or 6 days after the induction of sex change, and from control males and females in stable groups. For each tissue, we measured levels of estradiol (E2), testosterone (T) and 11-ketotestosterone (KT). Females had more E2 and T than males but there was no sex difference in KT. For both sexes, E2 was higher in the gonads while androgens were higher in the brain. Alpha females had lower brain KT than gamma females and lower gonadal E2 than beta females. During sex change, brain T levels dropped while KT increased, and E2 levels did not change. The results demonstrate that steroid levels are responsive to changes in the social environment, and that their concentrations vary in complex ways in different tissues. Also, rapid changes in brain androgen

levels might be implicated in inducing behavioral and/or morphological changes associated with protogynous sex reversal.

Introduction

Sex steroids have long being studied for their role in modulating social behavior, but these social interactions can, in turn, modulate steroid levels in the context of either reproduction (Liley et al., 1986) or aggression (Oliveira et al., 2002; Wingfield, 1984). For example, in the protogynous stoplight parrotfish, *Sparisoma viride*, simulated territorial intrusion for a week increases aggressive behavior and plasma androgen levels of resident males (Cardwell and Liley, 1991a). Contest duration and escalation affect post-fight steroid levels in the mangrove killifish (Earley and Hsu, 2008), and even just watching a fight between two other males increases androgen levels in the cichlid *Oreochromis mossambicus* (Oliveira et al., 2001b). In Gulf toadfish, *Opsanu beta,* the presence of a group of calling males or territorial challenges simulated by acoustic playbacks increase levels of 11-ketotestosterone (KT, Remage-Healey and Bass, 2005), the most potent fish androgen. The vast majority of studies on steroids and aggression focus on males, and when females are considered, results are not always concordant. In response to territorial intrusion, testosterone (T) levels decrease in female song sparrows (Elekonich and Wingfield, 2000) and they do not change in female California mice (Davis and Marler, 2003). In the cooperative breeding cichlid *Neolamprologus pulcher,* female and male residents and intruders have higher T and KT than controls that did not receive a territorial challenge, but only male aggression correlates with T (Desjardins et al., 2006). Estrogen levels were not affected by the social challenge (Davis and Marler, 2003; Desjardins et al., 2006; Elekonich and Wingfield, 2000).

All the studies noted above consider changes in steroids as measured from circulation. While the gonads remain an important source of steroids and probably a major contributor to circulating levels, there is evidence that the brain is also capable of steroidogenesis. Homogenates of brain tissues from goldfish, toadfish and Atlantic salmon parr produced large amounts of estrogen, and ovaries and testes synthesized much less estrogen than brain tissue (Andersson et al., 1988; Pasmanik and Callard, 1986). In fish, male blood KT levels are typically high, but there is evidence that this could be due to extragonadal production: after castration, implanting male sticklebacks with the KT precursor 11-ketoandrostenedione (OA) produced high plasma KT levels (Mayer et al., 1990b). In male rainbow trout, many different tissues were able to convert OA into KT: the highest activity was found in liver, testis and kidney, followed quite closely by intestine, brain and spleen, while muscle did not present any activity (Schulz and Blum, 1991). The presence of 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, which is an enzyme necessary to produce T, has even been documented in blood cells of various fish species (Mayer et al., 1990a; Schulz, 1986).

Local synthesis of steroids in the brain can have important effects on behavior through fast, non-genomic mechanisms that can act in a time frame more consistent with rapid behavioral changes (Balthazart and Ball, 2006; Cornil et al., 2006; Remage-Healey and Bass, 2006; Schmidt et al., 2008; Woolley, 2007). *In vivo* microdialysis of male zebrafinch brain, showed local changes in steroid levels in response to social stimuli: playback of male songs caused an increase in estradiol (E2) and a decrease in T, while presenting females to males caused an increase in E2 but not T (Remage-Healey et al., 2008). It is interesting to note that steroid concentrations changed in the brain but not in plasma. Some studies have found a correlation between plasma and tissue steroid levels, but few have tried to correlate levels across different tissues. For

example, in koi *Cyprinus carpo*, there is a strong correlation between blood KT and mucus KT, but not between blood and muscle (Schultz et al., 2005). In the ratfish *Hydrolagus colliei*, there is a significant positive correlation between plasma and muscle levels of T and KT, but not for E2 (Barnett et al., 2009). A study on the protogynous *Thalassoma duperrey* showed some discrepancies between gonad and plasma steroids (Nakamura et al., 1989). In fact, *in vitro* culture of portions of gonads showed that testes of terminal phase (TP) males produced much more KT than females and newly sex changed males, and gonads of sex changed males produced more than those of females. When comparing plasma though, neither TP males nor newly sex changed males differed from females. Testosterone secretion was higher in gonads of both TP and sex changed males relative to females, but there was no significant sex difference in plasma T levels (Nakamura et al., 1989).

Based on the studies summarized above, circulating steroid levels are not always concordant with tissue specific levels, so in the present study we examined how steroid levels in brain, gonad and muscle relate to each other and to sexual phenotype and social behavior. Specifically, we tested whether tissues show sex or status differences in steroid concentrations and whether they are responsive to changes in the social environment, in the bluebanded goby *Lythrypnus dalli*, a bidirectional sequential hermaphrodite. Sex change in this species is controlled by social status, and their small size (see below) allows for the expression of natural behavior and sex reversal in a laboratory setting. In *L. dalli*, females can be as aggressive as males and form linear dominance hierarchies, so it is also an ideal species for examining differences in steroids among females with different social status. Many studies have shown that sex steroid manipulations can affect sex change (reviewed by Frisch, 2004). As a very general summary, in protandrous species E2 levels increase with transition from male to female, and

administering E2 to males induces sex change; on the other hand, in protogynous species, KT levels increase during sex change, and KT can induce sex change in females; T in many species does not vary during sex change. The vast majority of these studies on sex change are based on levels of hormone measured from whole body homogenates (Kroon and Liley, 2000; Kroon et al. 2003) or from plasma (Bhandari et al., 2005; Bhandari et al., 2006; Cardwell and Liley, 1991b; Kobayashi et al., 1991; Lone et al., 2001; Ohta et al., 2008; Yeung and Chan, 1987) or released into water (Earley et al. unpublished data).

In the present experiment, we housed fish in groups to allow them to establish a stable social hierarchy, and we induced the start of sex change by removing the male from the group. We extracted steroids from brain, muscle and gonads of females of differing social status and, as we were interested in the role of steroid hormones as possible mediators of sex change, we focused on the early stages of sex change during the first week after male removal.

Materials and Methods

Lythrypnus dalli is a small benthic goby (20-45 mm adult standard length) that forms linear dominance hierarchies in a laboratory setting (Rodgers et al., 2007). Typically, large males dominate a nesting territory containing multiple females (Behrents, 1983). The fish used in this study were collected off the coast of Catalina Island, CA, in June 2007 by SCUBA diving (permit #803036-03 to VL), and were initially housed in communal holding tanks at the Wrigley Institute of Environmental Sciences. From these animals, thirty experimental social groups were established in indoor seawater tables with continuous seawater supply and exposure to the natural summer light cycle. Each social group consisted of a male (the largest animal in the tank), a large (alpha) female, a medium (beta) female that was at least 3 mm smaller than alpha,

to ensure a stable position in the hierarchy, and a small (gamma) female. Behavioral observations consisted of 10 min sessions during which we recorded approaches (a fish moving within one body length of another fish), submission (a fish is displaced or attacked by another), and aggression in the form of displacements (when a fish approaches and displaces another group member), attacks (a very quick and directed approach that ends in a displacement), and threat displays (a fish approaching another one, raising the dorsal fin and opening the opercula while swimming sideways).

In ten groups, fish were kept in the tank for 7 days without any social manipulation, and these are referred to as "stable" groups. Behavior was observed on day 6 in the morning and in the afternoon, and on day 7 in the morning. Tissue samples were collected from all the males and females in these groups. In the other twenty social groups, the male was removed from each tank after 6 days to induce the start of sex change in the alpha female. Behavior was observed on day 5 in the morning and in the afternoon, on day 6 in the morning and one hour after male removal in the afternoon, and on day 7 (the day after male removal) in the morning. In ten of these groups, tissues were collected from the alpha and beta females 24 hr after male removal, and these will be referred to as "sex change 24 hr" groups. In the remaining ten groups, tissues were collected from alpha and beta females 6 days after male removal. In these groups, additional behavioral observations were performed 5 days after male removal in the morning and in the afternoon, and 6 days after male removal in the morning. These will be referred to as "sex change 6 days" groups. For each fish, we recorded standard length, and captured a digital image of the genital papilla at the start and at the end of the experiment. The shape of the genital papilla is a clear indicator of the sex of the fish and when expressed as length/width ratio: a ratio of 1.2 or less is typical of female round papillae, while males have a thin pointed papilla with a ratio >

1.6, and transitional fish have intermediate values (Rodgers et al., 2007). We used AxioVision (Zeiss Inc.) imaging software to take papilla measurements from digital images. Brain, gonads and axial muscle were collected, and a digital image of the gonads was taken to confirm sex and reproductive status. Each tissue was put in a microcentrifuge vial and immediately frozen in liquid nitrogen. All the fish were sacrificed between 2 and 4 p.m. The work presented in Chapter 2 showed that *L. dalli* water-borne androgen levels peak in the morning and E2 levels peak in the evening (Lorenzi et al., 2008), so we assayed tissues when the values show the least variation to avoid differences due to diurnal rhythms. The tissue samples were shipped frozen to Georgia State University and stored at -80°C until processing for steroid content.

Steroid analysis

Steroid hormones were extracted from tissue following a solid-phase extraction protocol (modified from Newman et al., 2008). Each frozen tissue sample was weighed, transferred to borosilicate vials, and homogenized in 350 μl of buffer in an ice-cold water bath (0.1 M phosphate buffer for brain and gonad samples, 0.1 M borate buffer for muscle samples). After homogenization, 1.5 ml methanol was added to each sample, and they were quickly vortexed and returned to the ice bath. When all samples were homogenized, they were vortexed for 60 min and then stored at 4°C overnight. We repeated the same protocol for 2 control blank samples without any tissues: one containing 0.1 M phosphate buffer and one with 0.1 M borate buffer. The next day, samples were vortexed again for 20 min at room temperature, and centrifuged at 1027 x g for 10 min at 4°C. The supernatant was decanted, and 16 ml of water was added to dilute the methanol. The homogenized samples were connected through high purity tubing (Saint-Gobain Tygon) to Sep-Pak C18 columns (Waters) mounted onto a vacuum manifold. Before starting, the columns were primed by running twice 2 ml of methanol, and twice 2 ml of distilled water. After

drawing the samples through the columns, the columns were rinsed by running twice 2 ml of distilled water, and then hormones were eluted with 2 ml of methanol repeated twice. The eluted methanol was collect in borosilicate vials, and dried under a stream of nitrogen. The dried samples were re-suspended in 350.5 µl of a solution made of 95% EIA buffer and 5% ethanol. After vortexing for 15 min, they were stored at 4°C overnight. On the third day, samples were vortexed for 45 min. EIA kits (Cayman Chemicals, Inc.) for E2, KT and T were used to quantify hormone in the samples. Assay plates were read on a spectrophotometer (Opsys MR, Dynex Technologies) at a wavelength of 405 nm after 90 min of incubation.

Hormone validations

We generated a hormone pool for each of the tissue types (brain, muscle, gonad) using samples from 15 non-experimental animals. Hormone was extracted from the non-experimental tissues using the same protocol as described above; the hormone residues were re-suspended in 95% EIA buffer and 5% ethanol and combined to give a pool for each tissue type. The pool was serially diluted to determine parallelism with the standard curve. Briefly, 400 µl of pool was mixed with an equal volume of EIA buffer to give 1:2; 400 µl of 1:2 was mixed with an equal volume of EIA buffer to give 1:4, and so on until 1:64. Serial dilution curves for all hormones and tissue types were parallel to the standard curve (slope comparisons: Zar 1996, p. 355): estradiol, brain: t_{12}=0.016, p=0.988, gonad: t_{11}=-2.163, p=0.053, muscle: t_{12}=-0.065, p=0.949; testosterone, brain: t_{12}=0.232, p=0.821, gonad: t_{12}=0.157, p=0.878, muscle: t_{12}=0, p=1; 11-ketotestosterone, brain: t_{12}=0.2, p=0.845, gonad: t_{12}=0, p=1, muscle: t_{12}=0.021, p=0.984. We also validated the Cayman Chemicals, Inc. EIA kits with a recovery (cold spike) assay where the tissue-extracted pool was mixed with an equal volume of each kit standard. Expected concentrations were calculated based on the known concentration of the pool. The slopes of the

observed vs. expected hormone concentration regressions were all close to 1.0, indicating adequate recovery (estradiol: brain: $F_{1,6}$=9295.6, p<0.0001, β=1.086, min recovery=110%, gonad: $F_{1,6}$=725.1, p<0.0001, β=1.633, min recovery=90%, muscle: $F_{1,6}$=833.1, p<0.0001, β=1.291, min recovery=112%; testosterone: brain: $F_{1,6}$=1023.4, p<0.0001, β=0.758, min recovery=53%, gonad: $F_{1,6}$=107.7, p<0.0001, β=0.869, min recovery=78%, muscle: $F_{1,6}$=469.6, p<0.0001, β=0.935, min recovery=81%; 11-ketotestosterone: brain: $F_{1,6}$=124.4, p<0.0001, β=0.846, min recovery=93%, gonad: $F_{1,6}$=999.9, p<0.0001, β=0.818, min recovery=81%, muscle: $F_{1,6}$=487.1, p<0.0001, β=0.759, min recovery=93%). Seven plates were run for each hormone. Intrassay coefficient of variation for E2 ranged from 3.97% to 19.7% (median=9.55%) with an interassay coefficient of variation of 14.8%. Intrassay coefficient of variation for T ranged from 5.23% to 14.5% (median=5.5%) with an interassay coefficient of variation of 9.86%. Intrassay coefficient of variation for KT ranged from 1.6% to 13.9% (median=8.1%) with an interassay coefficient of variation of 11.2%. Detection limit of the EIA kits were 8 pg/ml for E2, 6 pg/ml for T, 1.3 pg/ml for KT.

Statistical analysis

For stable groups, we compared hormone levels among males and alpha, beta and gamma females. We could not perform a multivariate analysis because levels of E2, T, and KT in each fish are not independent, so we performed three separate ANOVAs for each hormone. We tested for normality and homogeneity of variance. To reach normality, T and E2 levels were log transformed and KT was square root transformed. When main effects were significant, we performed Tukey's HSD posthoc tests. Since the number of possible comparisons between all status classes and tissues were very large, but only a subset of them was relevant, we chose the comparisons *a priori* and ran linear contrasts. Since multiple tissues were extracted from the

same animals, significant p-values were corrected with the sequential Dunn-Sidak adjustment to account for compounding of Type I error because the variables were dependent. For sex changing groups, we performed two different analyses. In the first, we performed ANOVAs comparing hormone levels of sex-changing alphas at 24 hr and 6 days after male removal to alphas of stable groups, and betas in sex-changing groups to betas in stable groups. In the second analysis, we performed ANOVAs to compare alphas in the sex-changing groups to males, and we compared betas in the sex changing groups to alphas in stable groups. So, with the first analysis, we tested whether steroid profiles in sex-changing alphas deviated significantly from stable alphas, while in the second one, we tested whether steroid profiles in sex-changing alphas approached the profile of stable males. Paired t-tests were used to compare values of papilla ratio for each animal at the start versus the end of the experiment. We performed correlations between behavior and hormone levels and between hormones in different tissues. The ANOVAs and linear contrasts were performed with SAS (SAS Institute Inc.), while the correlation analyses were performed with JMP (SAS Institute Inc.).

Results

Genital papilla

As expected, none of the fish in stable groups changed sex. In fact, males in stable groups had an average final papilla length/width ratio not significantly different from initial values (paired t test: t_9=1.33, p=0.215; initial=3.28±0.20; final=3.50±0.15). The same was true for alpha females (paired t test: t_9=1.29, p=0.229; initial=1.09±0.04; final=1.12±0.04), beta females (paired t test: t_9=0.64, p=0.540; initial=1.09±0.04; final=1.12±0.04) and gamma females (paired t test: t_9=0.90, p=0.394; initial=1.13±0.07; final=1.16±0.03) in stable groups.

Alpha females in sex change treatments did not show any significant changes in the papilla ratio 24 hr after male removal (paired t test: t_8=1.39, p=0.202; initial=1.12±0.02; final=1.16±0.03), but the papilla showed a significant shift towards the male typical shape 6 days after male removal (paired t test: t_9=2.51, p=0.033; initial=1.17±0.04; final=1.38±0.07). Beta females in sex change treatments did not show any significant changes in the papilla ratio neither 24 hr after male removal (paired t test: t_8=0.67, p=0.523; initial=1.08±0.02; final=1.11±0.02) nor 6 days after male removal (paired t test: t_9=0.54, p=0.601; initial=1.16±0.03; final=1.18±0.02).

Stable groups

In the following sections because of the large number of comparisons, we will report only the significant effects. When the significant results of post hoc tests are mentioned, they always refer to Tukey's HSD and $p<0.05$. The full list of all the significant linear contrasts and their statistics are reported in the tables at the end of the chapter.

Testosterone

For all fish not undergoing sex change, T was significantly different between tissues (ANOVA: $p<0.0001$, $F_{2,105}$=148.24). Brain had more T than either muscle or gonad, but muscle and gonad did not differ (Fig. 3.1). There was a significant difference in T levels across status classes (ANOVA: p=0.0002, $F_{3,105}$=7.21). Beta females had more T than males, and gamma females had more T than males and alpha females. Linear contrasts between all females together versus males showed that there was no significant sex difference in brain and muscle, but ovaries had significantly higher T than testes. When analyzing linear contrasts for each female separately, the only significant difference was that gonads of gamma females had more T than gonads of males (Table 3.1).

11-Ketotestosterone

KT was significantly different between tissues (ANOVA: $p<0.0001$, $F_{2,106}=168.83$), with more KT in brain than muscle or gonad and no difference between muscle and gonad (Fig. 3.1). There was a significant difference in KT levels across status classes (ANOVA: $p=0.0011$, $F_{3,106}=5.77$), due to the fact that gammas had more KT than alphas. Beta and gamma brains had more KT than alphas, and gammas also had higher brain KT than males, but the only significant difference was the one between gammas and alphas (Table 3.1). When grouping all females together, there was no significant sex difference in KT in brain, gonad, or muscle.

Estrogen

Estrogen was significantly different across tissues (ANOVA: $p<0.0001$, $F_{2,104}=224.58$), with gonad having more E2 than either muscle or brain (Fig. 3.1), and brain having more than muscle. There was a significant effect of status (ANOVA: $p<0.0001$, $F_{3,104}=71.86$), with alpha, beta and gamma having more E2 than males. There was a significant status by tissue interaction (ANOVA: $p<0.0001$; $F_{6,104}=12.11$). Females had more E2 than males in gonad, brain, or muscle (Table 3.1). When examining each female separately, the only difference that was not significant was between alpha females and males in the brain (Table 3.1). In the gonad, beta and gamma females had more E2 than alpha females (Table 3.1). In females, gonads had more E2 than the other tissues, and brain had more than muscle, but in males gonad and brain did not differ, and they both had more E2 than muscle (Table 3.1).

Sex changing groups: Comparing alpha and beta females

In this statistical analysis, we compared hormone levels of alpha and beta females in stable groups with those of alpha and beta females at 24 hr and 6 days after male removal, respectively.

Testosterone

There was a significant difference in T across tissues (ANOVA: $p<0.0001$, $F_{2,149}=192.06$) with brain having more T than gonad or muscle (Fig. 3.2), and gonad more than muscle. There was also a significant difference in T levels across time (ANOVA: $p<0.0001$, $F_{2,149}=157.00$). Females in stable groups had higher T levels than those in sex changing groups, but there was no difference at 24 hr or 6 days after male removal. There was also a significant effect of status (ANOVA: $p=0.0003$, $F_{1,149}=13.41$) due to the fact that beta females had more T than alphas. Alpha and beta T levels did not differ in brain or muscle but betas had significantly more T in the gonad (Table 3.2). There was a significant interaction between time and tissue (ANOVA: $p<0.0001$; $F_{4,149}=24.74$). In gonads, T levels did not differ between stable groups and any of the sex changing groups. Stable groups had more T than 24 hr and 6 days in both brain and muscle (Table 3.2). Gonad and muscle did not differ in T levels in stable groups, and brain and gonad did not differ in 24 hr groups and 6 days groups. Beta females had more E2 in gonads compared to alpha females (Table 3.2).

11-Ketotestosterone

There was a significant difference in KT across tissues (ANOVA: $p<0.0001$, $F_{2,152}=342.45$) with brain having more KT than gonad or muscle (Fig. 3.2), and gonad more than muscle. There was a significant interaction between status and time (ANOVA: $p=0.0016$; $F_{2,152}=6.70$). Linear contrasts show that KT levels in alpha females were higher at 24 hr and 6 days after male removal than in stable groups (Table 3.2). There was also a significant interaction between tissue and time (ANOVA: $p<0.0001$; $F_{4,152}=8.66$). In fact, KT levels in the brain were significantly higher at 24 hrs and 6 days than in stable groups (Table 3.2).

Estrogen

Estrogen levels were significantly different across tissues (ANOVA: $p<0.0001$, $F_{2,143}=515.87$) with gonads having more than either brain or muscle (Fig. 3.2), and brain having more than muscle. There was a significant difference across time (ANOVA: $p=0.0453$, $F_{2,143}=3.16$) with females having higher E2 at 6 days after male removal than at 24 hr but no difference between females in stable groups and those in sex changing groups. There was a significant effect of status (ANOVA: $p=0.0006$, $F_{1,143}=12.41$) with betas having higher E2 than alphas. Linear contrasts show that betas had higher E2 than alphas in 6 days groups (Table 3.2) but alpha and beta E2 levels did not significantly differ in any of the tissues. There was a significant interaction between status and time (ANOVA: $p=0.0327$; $F_{2,143}=3.50$) because beta E2 was significantly higher at 6 days than at 24 hr (Table 3.2), but alpha females E2 levels did not differ across any sampling time.

There was a significant three-way interaction between status, time and tissue (ANOVA: $p=0.0097$; $F_{4,143}=3.47$) so we performed three-way linear contrasts (refer to Table 3.3 for a list of all the statistics). For alphas and betas within each treatment, brain had higher E2 than muscle, and gonad had higher E2 than both brain and muscle. The three-way interaction seemed to be driven largely by within status differences among tissue type. In fact, there were no differences over time or between status in E2 levels in brain and muscle but there were treatment differences in the gonad. In beta females, gonadal E2 was significantly higher at 6 days than at 24 hr. Beta gonadal E2 at 6 days was also significantly higher than alpha gonadal E2 in stable groups and in 6 days groups.

Sex changing groups: Comparing with males

In this statistical analysis, we compare hormone levels of alpha and beta females at 24 hr and 6 days after male removal with those respectively of males and alpha females in stable groups.

Testosterone

T levels were significantly different between tissues (ANOVA: $p<0.0001$, $F_{2,149}=194.91$), with brain having more T than gonad or muscle, and gonad more than muscle. There was also a significant difference in T levels across time (ANOVA: $p<0.0001$, $F_{2,149}=111.60$) with males and alphas in stable groups having more T than alphas and betas in both sex changing groups, but no difference between 24 hr and 6 days after male removal. There was also a significant difference between status classes (ANOVA: $p=0.0441$, $F_{1,149}=4.12$) with betas in sex changing groups and alphas in stable groups combined having more T than alphas in sex changing groups and males combined. There were significant interactions between status and tissue (ANOVA: $p=0.0378$; $F_{2,149}=3.35$) and time and tissue (ANOVA: $p<0.0001$; $F_{4,149}=29.01$). Males had higher T levels compared to sex changing alphas both at 24 hr and at 6 days (Table 3.4). Alphas in stable groups had higher T than beta females in both 24 hr and 6 days groups (Table 3.4).

11-Ketotestosterone

There was a significant difference in KT across tissues (ANOVA: $p<0.0001$, $F_{2,152}=311.18$) with brain having more KT than gonad or muscle, and gonad more than muscle. There was no significant difference in KT levels across time between stable and sex changing groups (ANOVA: $p=0.0687$, $F_{2,152}=2.73$). KT was different between status classes (ANOVA: $p=0.0005$, $F_{1,152}=12.53$) with alphas in sex changing groups and males combined having more T than betas in sex changing groups and alpha in stable groups combined (Table 3.4). The interaction between tissue and time was significant (ANOVA: $p<0.0001$; $F_{4,152}=8.93$). Linear contrasts show that

males did not differ in KT levels from any sex changing alpha females, and beta females from sex changing groups did not differ in KT levels from alphas from stable groups (Table 3.4).

Estrogen

Estrogen levels were significantly different across tissues (ANOVA: $p<0.0001$, $F_{2,145}=357.27$) with gonads having more E2 than either brain or muscle, and brain having more than muscle. There was a significant difference across time (ANOVA: $p<0.0001$, $F_{2,145}=52.22$) with fish in both the sex changing groups having more E2 than in stable groups. There was also a significant effect of status (ANOVA: $p<0.0001$, $F_{1,145}=89.97$) with betas in sex changing groups and alpha in stable groups combined having more T than alphas in sex changing groups and males combined. There were significant interactions between status and tissue (ANOVA: $p=0.0007$; $F_{2,145}=7.71$), between status and time (ANOVA: $p<0.0001$; $F_{2,145}=30.35$), and between time and tissue (ANOVA: $p<0.0001$; $F_{4,145}=13.22$). Males had significantly lower E2 than both sex changing alphas at 24 hr and 6 days (Table 3.4). Alphas in stable groups had significantly lower E2 levels than beta females at 6 days (Table 3.4), but alphas did not differ from beta females at 24 hr.

There was a significant 3-way interaction between status, time and tissue (ANOVA: $p=0.0002$; $F_{4,145}=5.86$) so we performed 3-way linear contrasts (refer to Table 3.5 for a list of all the statistics). For alphas and betas within each treatment, brain had higher E2 than muscle, and gonad had higher E2 than both brain and muscle. The three-way interaction seemed to be driven largely by within status differences among tissue type; in fact, there were no differences over time or between status in E2 levels in brain and muscle but there were treatment differences in the gonad. Male E2 levels in gonad and muscle was significantly lower than concentrations of alpha and beta females in all treatments. In the brain, male E2 was significantly lower than beta

in stable and 6 days groups, and showed a trend for values lower than alpha females in stable and 6 days groups, and lower than beta at 24 hr. Alpha females gonadal E2 was significantly lower than 6 days beta females.

Hormone by hormone correlations

We correlated the concentrations of each steroid in each tissue with the concentration of every other steroid, due to the large number of comparisons, we report all the detailed statistics in Table 3.6. When comparing all the fish together (Fig. 3.3), KT in the gonad correlated with KT in the brain and with T in the gonad, KT in the muscle correlated with T in the muscle, and E2 in the muscle with E2 in the brain. When considering each sex separately, in females the same correlations were significant except for the one for E2, and in addition, gonad KT negatively correlated with muscle T. In males, there was only one significant correlation between KT and T in the muscle. When analyzing each class of female separately in stable groups, the only significant correlation recurring in all female categories was between KT and T in the muscle and KT and T in the gonad (Fig. 3.4). In alpha females there was also a negative correlation between gonad E2 and muscle E2. In gamma females there was a correlation between brain T levels and T and KT in the gonad.

In sex changing alphas at 24 hours after male removal, there was only a significant correlation between KT in muscle and gonad (Fig. 3.4). At 6 days after male removal, alphas show a positive correlation between muscle T and brain E2, and negative correlations between muscle T and gonad KT, and between gonad KT and brain E2 (Fig. 3.5). They also showed a positive correlation between gonad E2 and brain E2, and negative between gonad E2 and gonad KT (Fig. 3.5). Betas in all sex changing groups showed the correlation between KT and T in both muscle and brain. In addition, in betas at 24 hours, there were significant positive correlations

between brain E2 and gonad E2 (Fig. 3.6) and gonad KT and gonad T. In betas at 6 days, KT and T also positively correlated in the gonad and in the brain (Fig. 3.6), and their gonad KT also correlated with muscle KT and T.

Hormone by behavior correlations

We correlated the steroid concentration in each tissue type with all the behavioral categories (approaches, attacks, displacements, courtship, threat displays and displacement received), and the full list of significant correlations and their statistics is presented in Table 3.7. In stable groups, only in males there was a significant negative correlation between E2 in the gonad and total number of approaches and displacements.

In 24 hr sex changing groups, gonad and muscle KT of alpha females positively correlated with approaches measured either 1 hr after male removal or as a total of all the observations made after male removal (Fig. 3.7). In 6 days groups, alpha E2 in the muscle positively correlated with attacks, approaches, and displacements measured 1 hr after male removal, while brain E2 correlated with attacks observed 1 day after male removal (Fig. 3.7). Muscle KT positively correlated with the total of displacements observed after male removal while gonad KT negatively correlated with the total of approaches observed before male removal.

For beta females, brain T and KT positively correlated with displacements received on the first day after male removal in 24 hr sex changing groups, but they negatively correlated with displacements received on the fifth day after male removal in 6 days sex changing groups (Fig. 3.8). There were a few more significant correlations in betas in 6 days groups: gonad E2 correlated with total aggression measured before and after male removal, and with displacements given before male removal (Fig. 3.8). Gonad KT correlated with total displacements received

after male removal, and negatively with approaches given on the afternoon of the fifth day after male removal. Gonad T negatively correlated with total aggression and displacements given 5 days after male removal.

Discussion

Our results show that different tissues can have very different steroid levels, and therefore caution is required when using plasma or whole body values to predict local levels. We also confirmed the presence of sex differences in E2 and T levels but KT, that is considered a potent male androgen, does not differ among the sexes. The data also suggest that the gonad in *L. dalli* is the main source of estrogen and that the brain is an important source of androgens supporting the idea that local synthesis gives a relevant contribution to brain steroid levels. We demonstrated that during sex change different tissues react differently to the same social manipulation, showing how different organs can be independently regulated. We do not find any strong correlation between any hormones and behavior so in an animal with a complex social structure as *L. dalli*, behaviors are not easily reducible to the influence of a single hormone.

Sex differences in hormone levels

Our data show that *L. dalli* females have more T than males in the gonads, and more E2 and similar KT in brain, gonad and muscle. Higher estrogen levels in females are quite typical in vertebrates but sex differences in androgens show more variability across different taxa. In mammals, males have higher androgens than females but in fish females often have as much T as males (Borg, 1994). KT is considered a potent male androgen in fish, and is most typically higher in males but there are instances of sex changing species that show no sex difference (Borg, 1994). The data presented in Chapter 2 measuring circulating levels of steroids from

water samples in *L. dalli*, found higher E2 in females and no sex differences in T and KT (Lorenzi et al., 2008). The sex differences we now found for E2 in tissues are consistent with that study and with another one showing that in *L. dalli* aromatase activity is higher in females than males (Black et al., 2005a). In contrast to the water samples, we found that gamma female gonads had significantly more T than males so maybe this gonadal sex difference might be obscured when you do not separate the females based on social status or you consider circulating levels instead of tissue-specific levels. The lack of sex difference in KT in *L. dalli* might be associated with the fact that this species shows little sexual dimorphism and maintains very high degree of sexual plasticity throughout its life by being able to change sex in both directions.

Different tissues present different concentrations of steroids

The brain has higher E2 levels than muscle but not different from testes, and ovary has much more E2 than both brain and muscle. This might not seem surprising since ovaries are an important site of estrogen synthesis (Devlin and Nagahama, 2002), but it is in contrast with other teleosts in which aromatase activity is typically reported to be higher in brain than gonad (Li et al., 2007; Pasmanik and Callard, 1985). Our present results on levels of E2 are consistent with previous studies from our laboratory showing much higher aromatase activity in gonad than brain of female *L. dalli* (Black et al., 2005a). Beta females have significantly more E2 than alpha females in the gonad but there are no differences in E2 in brain and muscle across females with different social rank so E2 levels seem to be associated more with sexual function than status. Our results for E2 are consistent with a study on rat that showed no difference in E2 concentrations between testis, brain and muscle but higher E2 in the ovary (Aizawa et al., 2007).

The results for androgens were very unexpected: both T and KT are much higher in the brain than in gonads and muscle. Possible explanations are that the brain is an important site of

androgen synthesis or that it sequesters androgens from circulation. Brain homogenates from various fish species can convert androstenedione to T and E2 (Pasmanik and Callard 1985; Andersson et al 1988), and in male rainbow trout brain tissue was able to convert OA to KT (Schultz and Blum 1991), so *in vivo* production of androgens could be significant. In contrast with our results, male rainbow trout testis had higher 17β-HSD activity than brain (Schultz and Blum 1991), so there could be differences in relative hormone concentrations between gonochoristic and hermaphroditic species. The hormone levels we report are the sum of all steroids present in the tissue at that moment. This includes what has been synthesized there, what has arrived from circulation, and what is bound to proteins. So one alternative explanation is that the brain has more androgen receptors than gonad and muscle and therefore is binding more T and KT. In the protogynous wrasse *Halichoeres trimaculatus*, there was no sex difference in the expression of estrogen (ER) or androgen receptors (AR) in the gonad but TP males expressed more AR in the brain than females and initial phase males (Kim et al., 2002).

We found no significant difference in T between gonad and muscle. The T present in the muscle could be coming from the gonad through circulation or be produced there. There is evidence in rats that muscle can produce both T and E2 (Aizawa et al., 2007), but a study screening various tissues for 17β-HSD activity in male rainbow trout did not find any in the muscle (Schultz and Blum 1991). Our results for T are in contrast with those from rat, where ovary and testis presented more T than both brain and muscle (Aizawa et al., 2007). When considering only muscle, male rats have more T but there is no sex difference for E2 (Aizawa et al., 2008) while we found the opposite in *L. dalli* muscle: no sex difference for T and lower E2 in males. In mammals, circulating T is much higher in males than females, so the strong

contribution of gonadal steroids coming from circulation might explain the higher T muscle levels in males.

It is interesting to note that alpha females have values of both E2 and T intermediate between the low levels of males and the high levels of subordinate females, so they might already be in an hormonal state that is conducive or preliminary to sex change.

Estrogen does not change after male removal

After the removal of the male from the social groups, we observed changes in steroid levels in tissues of both alpha and beta females. For alpha females it is difficult to partition the effects due to change in social status from those due to sex change, but comparing the steroid response with that of beta females that are not going through sex change provides some important insights. In alpha females, E2 levels do not change significantly during the first 6 days after male removal. In beta females, E2 is higher at 6 days after male removal than at 24 hr but there is no difference compared with stable groups. Six days after male removal, sex changing alphas still have E2 levels much higher than males in all three tissues, and this can be explained by the fact that it takes longer than 6 days to reabsorb all the eggs and ovarian tissue. Previous work on *L. dalli* showed that aromatase activity decreases during the first stages of sex change in the brain but not in the gonad (Black et al. 2005a). This result matches with our finding for the gonad but not for the brain. A possible explanation is that even if there is a decrease in brain aromatase activity, we do not detect any changes in brain E2 levels because it could still be coming from the gonad in large amounts. In fact, as we mentioned above, in *L. dalli* the gonad has much higher aromatase activity than the brain (Black et al., 2005a).

KT increases after male removal

KT seems to be the hormone that distinguishes the endocrine response of the sex changing fish from the others. Specifically, KT in alphas increases at 24 hr and 6 days, while it does not significantly change in betas in any of the tissues. In *L. dalli*, KT can induce the masculinization of the genital papilla (Carlisle et al., 2000) so the increase in alpha KT levels is consistent with a male typical papilla ratio in alpha females at 6 days. The rise in KT during sex change might be needed to induce the start of spermatogenesis or the onset of male typical behavior, but is not necessary for the maintenance of male function because in stable groups we found no sex difference. This result is similar to what was found in stoplight parrotfish, *Sparisoma viride*, where serum KT increased in females during sex change into initial phase (IP) males, and increased again when changing color from IP into TP (Cardwell and Liley, 1991b). In the same study, injections of KT induced the start of gonadal sex change, transitional coloration, and depressed levels of E2. The increase in KT might have the primary function to inhibit E2 production because in the absence of E2 oocytes regress allowing gonadal sex change to proceed. In the honeycomb grouper *Epinephelus merra*, females that were given KT implants became males and had higher KT and lower E2 than vehicle controls (Bhandari et al., 2006). Maybe KT is able to stimulate sex change without any artificial manipulation of E2, by inhibiting its synthesis. Since KT levels increase before the testis develops, then extra-gonadal synthesis seems to be essential to initiate the process. Blood cells of salmon (Mayer et al., 1990a) and rainbow trout (Schulz, 1986) incubated with the precursor OA, presented conversion into KT so this synthesis could contribute to a rapid increase in circulating KT levels. On the other hand, an *in vitro* study on the gonads of *E. merra* (Alam et al., 2006) localized the cytochrome P450-11beta hydroxylase (P450-11beta), a key enzyme for the synthesis of KT, in the tunica near the

blood vessels of the ovary. The presence of P450-11beta in the female gonad could be responsible for both the low level of constitutive synthesis of ovarian KT and the increase in KT that blocks E2 synthesis during sex change. Interestingly, in stable groups alpha females had the lowest levels of KT in all tissues and this might make the rapid and sustained increase in brain and gonadal KT that happens during sex change more effective.

T drops after male removal

In *L. dalli*, T drops in brain and muscle of both alpha and beta females after male removal, suggesting an association with the lack of a male, rather than the induction of sex change *per se*. Presentation of a female can cause a rapid increase in male plasma T in rodents (Kamel et al., 1975; Macrides et al., 1975), and this is thought to facilitate mating. In the same way, the presence of an animal of the opposite sex might increase T levels in *L. dalli*. The drop in T might act as a chemical message to signal the lack of mating opportunity, but it is not sufficient to induce sex change without the increase in KT that only occurs in the new dominant animal. There are contrasting results on the effect of T manipulations on sex change (Frisch, 2004) but our data suggest that it would be interesting to inhibit it instead of increasing its levels to try to induce sex change. Work on stickleback has shown that T and KT can bind to the same androgen receptor with the same affinity but KT has higher efficiency in activating the receptor (Olsson et al., 2005). Since in stable groups T levels are orders of magnitude higher than KT, a drop in T might be necessary to allow KT to bind to the androgen receptors and initiate events that lead to behavioral and morphological changes.

Another possibility is that androgens are sequestered from circulation, and accumulated in the brain at a concentration higher than the gonadal source by binding to steroid binding globulins (SBG). In fact, SBG can be involved in the recognition and penetration of steroid

hormones from the circulation into the target cells (Avvakumov et al., 1986), and in rats it has been shown *in vitro* and *in vivo* that SBG can be internalized by neurons (Caldwell et al., 2007). In goldfish, zebrafish and seabass, SBG affinity for KT was about 10-fold less than for testosterone (Miguel-Queralt et al., 2005; Miguel-Queralt et al., 2004; Pasmanik and Callard, 1986) so KT can easily be displaced by an excess of T.

Previous work in our laboratory has measured steroids from water samples during sex change and showed a tendency for both T and KT to rise and for E2 to fall after 3 or 7 days following male removal (Earley et al., unpublished data). The results for KT are consistent with the present findings while those for T and E2 are not. The difference in E2 response might be simply an issue of timing: many of our alpha females had very ripe ovaries so that might have prolonged the high rate of E2 synthesis. We did not have any groups in tanks for more than 6 days after male removal, so we would have missed any changes that begin after our six day samples. A final difference between water and tissue comes from T, because we see a big drop in brain but not water concentrations, so water levels might primarily reflect what happens in the gonad.

Very few hormone by hormone correlations

We correlated the steroid concentrations in each tissue with the concentration of every other steroid for all the fish together, for male and females separately and for each class of female. We do not discuss all comparisons here, but you can refer to the result section and the tables for a full list. Since fish were undergoing different social situations and changing steroid levels in different ways, we found it most informative to analyze each class of fish separately. We were surprised to find very few significant correlations when comparing the same hormone across different tissues within individuals. One might expect that if one tissue represents the main

source of steroid, and if the steroids are completely free to reach the other organs through circulation, then there should be a correlation in steroid levels across tissues within individuals. For example, fish whose gonad is producing high levels of E2, would also have more E2 in brain and/or muscle. Instead, our results suggest that each tissue is remarkably independent with regard to steroid concentrations, possibly by having different rates of steroid synthesis and/or catabolism or through binding to different types/concentrations of steroid receptors.

KT correlates with T but not in the brain

The only strong correlation that held true for all females in stable groups was between KT and T in both muscle and gonad. Similarly, in male *Tilapia zillii*, plasma KT was positively correlated with plasma T (Neat and Mayer, 1999). This could be explained by the fact that T is a precursor of KT so higher T concentrations might lead to higher KT synthesis. For the same reason though, we would expect to see the same relationship in the brain but we find it only in betas of sex changing groups. Schultz and Blum (1991) suggest that high levels of steroids like T with high affinity for SBG might increase KT production by displacing its precursor OA from the globulin and making it available to synthetic enzymes, and so this could also explain the correlation we found. **A**n alternative explanation is that the animals that show higher androgen levels have more androgen receptors in those tissues and therefore they are binding more of both T and KT. Maybe there is no correlation in the brain because it has a different subtype of androgen receptor that selectively binds to KT and T with different affinities.

KT and T do not correlate in sex changing fish

It is worth noting that in alphas undergoing sex change there is a lack of the strong relationship between KT and T that was present in the other females. These are also the fish that are showing a rapid increase in KT levels so maybe a different mechanism is engaged to produce these rapid

changes, or most of the precursor T is quickly used to produce KT. In alphas 24 hr after male removal, there was only a correlation between KT in muscle and KT in gonad. This could be interpreted as the more KT is produced by the gonad (or the brain), the more ends up in the muscle, or that when KT synthesis is up-regulated in an organism, it happens in multiple tissues. At 6 days after male removal, alphas show a negative correlation between gonad KT and brain E2 and between gonad KT and gonad E2. As we mentioned above, KT has been suggested to inhibit E2 production in other species: fish treated with aromatase inhibitor had higher KT levels (Bhandari et al., 2005), and those treated with KT implants had significantly lower E2 levels than controls (Bhandari et al., 2006).

Betas in all sex changing groups showed the correlation between KT and T in muscle and brain, and in the gonad at 6 days after male removal, so their steroid pattern is more similar to the fish in stable groups. This makes sense because they are not changing sex, but at the same time, betas do show changes in steroid levels in response to their new social situation.

E2 does not correlate with T

As we found correlations between levels of KT and its precursor T, we also expected some correlations between T and another of its products, E2. In the cooperative breeding cichlid *N. pulcher,* levels of T correlated with both E2 and KT in plasma (Desjardins et al., 2006). On the other hand, in the protandrous *Amphiprion melanopus*, T correlated with E2 in females but not in males, and none of the correlations were present in fish in the process of changing sex (Godwin and Thomas, 1993). In *L. dalli*, we did not find a correlation between T and E2 in any of the individuals or tissues, which suggests that E2 could be synthesized using an alternative pathway. In the protogynous wrasse *Pseudolabrus sieboldi*, E2 is produced via estrone by aromatization of

androstenedione instead of T (Ohta et al., 2001). Further work will be needed to test whether this happens in *L. dalli* too.

Androgens do not correlate with aggression

In terms of the relationship between hormones and behavior, no overall pattern emerged, and correlations, if present, were different across treatments and social classes. We do not find, in *L. dalli*, any clear connection between androgens and aggression levels. For alpha females in sex changing groups at 24 hours, gonad and muscle KT positively correlated with the total rate of approaches to other fish but not with measures of overt aggression such as displacements. The vast majority of studies finding a relation between androgens and aggression use the resident/intruder paradigm while in our experiment the fish already coexist. This could explain the different results because the hormonal and/or behavioral response might be less dramatic when all fish are resident. In groups sacrificed at 6 days after male removal, muscle E2 of alpha females positively correlated with aggressive behaviors measured 1 hour after male removal, while brain E2 correlated with attacks observed 1 day after male removal. For beta females in 6 days groups, gonad E2 correlated with total aggression measured before and after male removal. Maybe in female *L. dalli* estrogens rather than androgens are associated with aggression but further evidence is needed to confirm this possibility. On the day of male removal, the sex changing alpha females often show increased aggression (Reavis and Grober, 1999) but then it goes back to lower levels similar to those of stable groups. The few significant correlations we found involve the behavior after male removal, so sex steroids might play a role only in situations of social instability as suggested by the "challenge hypothesis" (Wingfield, 1984). This could explain why we do not see any correlations between hormones and behavior in females in stable groups. We should also mention that *L. dalli* is not the only species where KT has no

effect on aggression. For example, sneaker males of the peacock blenny *Salaria pavo* implanted with KT showed no difference in a test of aggression against a mirror (Oliveira et al., 2001a). In male *Tilapia zillii*, immediately after fighting there was no differences in T or KT between losers, winners and controls that did not fight (Neat and Mayer, 1999). In *N. pulcher,* a territorial challenge increased androgens in both females and males but T correlated with aggression only in males and in residents while neither T, KT nor E2 correlated with aggression in females or intruders (Desjardins et al., 2006).

One last important difference between ours and other studies that find an effect of androgens on social status, is that in our social groups all animals are reproductively active. In the rainbow trout *O. mykiss,* males that became dominant increased their T level in laboratory tanks. However, under field conditions, there was no difference in T and KT between dominant and subordinate males of brook trout *Salvelinus fontinalis*. The authors suggest that the difference is probably due to the fact that subordinates do not spawn in the tank while they engage in sneak spawning in the field (Cardwell et al., 1996). As for many studies, results obtained from an artificial social situation in a laboratory setting might not be representative of the more subtle effects and more complex interactions that take place in the wild. We have the advantage that we can recreate in the laboratory tank whole social groups of spawning bluebanded gobies that have interactions similar to natural groups.

Conclusions

To summarize, our results show that different tissues within the same animal can have very different steroid levels, and that they can react differently to the same social stimulus so measuring circulating levels might not provide enough information in terms of local effects. The only recurring strong hormone correlation is between T and KT levels in gonad and in muscle

while most of the other correlations are specific to only one social status or treatment. The data suggest that the gonad in *L. dalli* is the main source of estrogen and that the brain is an important source of androgens but future studies should seek to determine whether these tissue differences are due to differential rates of synthesis/catabolism or active compartmentalization by steroid binding globulins or steroid receptors. Even if female *L. dalli* have more E2 than males in all tissues analyzed and more gonadal T, androgens, rather than estrogen, seem to be the candidate to translate the changes in the social environment that initiate sex change. In fact, during the first 6 days of sex change, E2 levels stay stable in all tissues, T decreases dramatically in brain and muscle, and KT increases in the brain, so we propose that an increase in brain KT with an associated drop in T is the steroidal milieu that initiates sex change and might have an inhibitory effect on estrogen synthesis. Socially regulated sex change provides an excellent model to study how an organism can differentially modulate steroid concentrations in tissues, and further our understanding of the action of the ubiquitous and essential hormones that are the sex steroids.

Table 3.1: List of all linear contrasts that are significant or show a trend towards significant values for fish in stable groups.

Contrast	p-value, F ratio	k	Adjusted α	Significant after adjustment?
Testosterone				
Male brain > male gonad	$p<0.0001$, $F_{1,105}=94.16$	33	0.00155	Y
Male brain > male muscle	$p<0.0001$, $F_{1,105}=60.47$	33	0.00160	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,105}=52.37$	33	0.00165	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,105}=58.26$	33	0.00171	Y
Gamma brain > gamma gonad	$p<0.0001$, $F_{1,105}=51.82$	33	0.00177	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,105}=47.44$	33	0.00183	Y
Gamma brain > gamma muscle	$p<0.0001$, $F_{1,105}=42.83$	33	0.00190	Y
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,105}=39.79$	33	0.00197	Y
Gamma gonad > male gonad	$p=0.0006$, $F_{1,105}=12.60$	33	0.00205	Y
Female gonad > male gonad	$p=0.0012$, $F_{1,105}=11.11$	33	0.00213	Y
Beta gonad > male gonad	$p=0.0045$, $F_{1,105}=8.43$	33	0.00223	N
Gamma muscle > male muscle	$p=0.0438$, $F_{1,105}=4.16$	33	0.00233	N
Gamma gonad > alpha gonad	$p=0.0690$, $F_{1,105}=3.38$	33	0.00244	N/A
11-Ketotestosterone				
Gamma brain > beta muscle	$p<0.0001$, $F_{1,106}=100.73$	33	0.00155	Y
Gamma brain > beta gonad	$p<0.0001$, $F_{1,106}=92.19$	33	0.00160	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,106}=78.57$	33	0.00165	Y
Male brain > male muscle	$p<0.0001$, $F_{1,106}=67.16$	33	0.00171	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,106}=60.95$	33	0.00177	Y
Male brain > male gonad	$p<0.0001$, $F_{1,106}=41.42$	33	0.00183	Y

Table 3.1: List of all linear contrasts that are significant or show a trend towards significant values for fish in stable groups (continued).

Alpha brain > alpha muscle	$p<0.0001$, $F_{1,106}=41.06$	33	0.00190	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,106}=34.62$	33	0.00197	Y
Gamma Brain > alpha Brain	$p<0.0001$, $F_{1,106}=19.77$	33	0.00205	Y
Beta Brain > alpha Brain	$p=0.0100$, $F_{1,106}=6.88$	33	0.00213	N
Gamma Brain > male Brain	$p=0.0115$, $F_{1,106}=6.61$	33	0.00223	N
Male brain > alpha brain	$p=0.0547$, $F_{1,106}=3.77$	33		N/A
Gamma brain > beta brain	$p=0.0638$, $F_{1,106}=3.51$	33		N/A
Estradiol				
Female gonad > male gonad	$p<0.0001$, $F_{1,104}=202.95$	33	0.00155	Y
Beta gonad > beta muscle	$p<0.0001$, $F_{1,104}=187.52$	33	0.00160	Y
Beta gonad > male gonad	$p<0.0001$, $F_{1,104}=175.72$	33	0.00165	Y
Gamma gonad > gamma muscle	$p<0.0001$, $F_{1,104}=145.48$	33	0.00171	Y
Gamma gonad > male gonad	$p<0.0001$, $F_{1,104}=141.91$	33	0.00177	Y
Beta gonad > beta brain	$p<0.0001$, $F_{1,104}=100.63$	33	0.00183	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,104}=94.63$	33	0.00190	Y
Alpha gonad > male gonad	$p<0.0001$, $F_{1,104}=94.61$	33	0.00197	Y
Gamma gonad > gamma brain	$p<0.0001$, $F_{1,104}=76.98$	33	0.00205	Y
Female muscle > male muscle	$p<0.0001$, $F_{1,104}=50.99$	33	0.00213	Y
Alpha gonad > alpha brain	$p<0.0001$, $F_{1,104}=45.70$	33	0.00223	Y
Male brain > male muscle	$p<0.0001$, $F_{1,104}=42.54$	33	0.00233	Y
Male gonad > male muscle	$p<0.0001$, $F_{1,104}=36.32$	33	0.00244	Y
Alpha muscle > male muscle	$p<0.0001$, $F_{1,104}=36.31$	33	0.00256	Y
Gamma muscle > male muscle	$p<0.0001$, $F_{1,104}=34.54$	33	0.00270	Y
Beta muscle > male muscle	$p<0.0001$, $F_{1,104}=31.24$	33	0.00285	Y
Beta brain > beta muscle	$p=0.0001$, $F_{1,104}=13.41$	33	0.00301	Y
Gamma brain > gamma muscle	$p=0.0005$, $F_{1,104}=10.81$	33	0.00320	Y

Table 3.1: List of all linear contrasts that are significant or show a trend towards significant values for fish in stable groups (continued).

Female brain > male brain	p=0.0009, $F_{1,104}$=9.69	33	0.00341	Y
Beta brain > male brain	p=0.0033, $F_{1,104}$=7.45	33	0.00366	Y
Gamma gonad > alpha gonad	p=0.0035, $F_{1,104}$=4.78	33	0.00394	Y
Alpha brain > alpha muscle	p=0.0036, $F_{1,104}$=7.33	33	0.00427	Y
Gamma brain > male brain	p=0.0044, $F_{1,104}$=6.99	33	0.00465	Y
Beta gonad > alpha gonad	p=0.0051, $F_{1,104}$=12.45	33	0.00512	Y
Alpha brain > male brain	p=0.0160, $F_{1,104}$=4.95	33	0.00568	N

P-values shown are uncorrected. The adjusted α value is based on the number of possible comparisons subjected to sequential Dunn-Sidak adjustments $\{1-(1-\alpha)^{1/k}\}$ where 'k' is the comparison number for the particular correlation being adjusted. To remain significant after adjustment, the uncorrected p-value must be less than the adjusted α value.

Table 3.2: List of all linear contrasts that are significant or show a trend towards significant values for sex changing groups.

Contrast	p-value, F ratio	k	Adjusted α	Significant after adjustment?
Testosterone				
Stable muscle > 6 days muscle	$p<0.0001$, $F_{1,149}=211.39$	36	0.00142	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,149}=193.85$	36	0.00146	Y
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,149}=190.88$	36	0.00151	Y
Stable muscle > 24 hrs muscle	$p<0.0001$, $F_{1,149}=166.70$	36	0.00155	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,149}=152.71$	36	0.00160	Y
Beta stable > beta 6 days	$p<0.0001$, $F_{1,149}=142.35$	36	0.00165	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,149}=141.47$	36	0.00171	Y
Stable brain > 6 days brain	$p<0.0001$, $F_{1,149}=123.80$	36	0.00177	Y
Stable brain > stable gonad	$p<0.0001$, $F_{1,149}=119.64$	36	0.00183	Y
Alpha stable > alpha 6 days	$p<0.0001$, $F_{1,149}=128.64$	36	0.00190	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,149}=110.97$	36	0.00197	Y
Beta stable > beta 24 hrs	$p<0.0001$, $F_{1,149}=103.77$	36	0.00205	Y
Stable brain > 24 hrs brain	$p<0.0001$, $F_{1,149}=102.92$	36	0.00213	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,149}=94.11$	36	0.00223	Y
Alpha stable > alpha 24 hrs	$p<0.0001$, $F_{1,149}=90.54$	36	0.00233	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,149}=90.44$	36	0.00244	Y
Beta gonad > beta muscle	$p<0.0001$, $F_{1,149}=77.73$	36	0.00256	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,149}=48.54$	36	0.00270	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,149}=45.95$	36	0.00285	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,149}=24.54$	36	0.00301	Y
Beta gonad > alpha gonad	$p=0.0021$, $F_{1,149}=9.76$	36	0.00320	Y
Stable gonad > 6 days gonad	$p=0.0071$, $F_{1,149}=7.45$	36	0.00341	N

Table 3.2: List of all linear contrasts that are significant or show a trend towards significant values for sex changing groups (continued).

6 days brain > 6 days gonad	$p=0.0342$, $F_{1,149}=4.57$	36	0.00366	N
Beta stable > alpha stable	$p=0.0542$, $F_{1,149}=3.77$	36		N/A
11-Ketotestosterone				
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,152}=330.16$	36	0.00142	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,152}=323.96$	36	0.00146	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,152}=303.06$	36	0.00151	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,152}=266.77$	36	0.00155	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,152}=172.82$	36	0.00160	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,152}=156.57$	36	0.00165	Y
6 days brain > 6 days gonad	$p<0.0001$, $F_{1,152}=149.88$	36	0.00171	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,152}=109.29$	36	0.00177	Y
24 hrs brain > 24 hrs gonad	$p<0.0001$, $F_{1,152}=96.10$	36	0.00183	Y
Stable brain > stable gonad	$p<0.0001$, $F_{1,152}=87.89$	36	0.00190	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,152}=39.44$	36	0.00197	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,152}=31.60$	36	0.00205	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,152}=28.35$	36	0.00213	Y
Beta gonad > beta muscle	$p<0.0001$, $F_{1,152}=23.55$	36	0.00223	Y
6 days brain > stable brain	$p<0.0001$, $F_{1,152}=17.64$	36	0.00233	Y
24 hrs brain > stable brain	$p=0.0003$, $F_{1,152}=13.57$	36	0.00244	Y
Alpha 24 hrs > alpha stable	$p=0.0005$, $F_{1,152}=12.80$	36	0.00256	Y
Alpha 6 days > alpha stable	$p=0.0005$, $F_{1,152}=12.72$	36	0.00270	Y
Stable muscle > 6 days muscle	$p=0.0036$, $F_{1,152}=8.76$	36	0.00285	N
Alpha 6 days > beta 6 days	$p=0.0055$, $F_{1,152}=7.94$	36	0.00301	N
24 hrs gonad > stable gonad	$p=0.0072$, $F_{1,152}=7.42$	36	0.00320	N
Stable muscle > 24 hrs muscle	$p=0.0075$, $F_{1,152}=7.34$	36	0.00341	N
Beta stable > alpha stable	$p=0.0263$, $F_{1,152}=5.04$	36	0.00366	N

Table 3.2: List of all linear contrasts that are significant or show a trend towards significant values for sex changing groups (continued).

Beta 24 hrs > alpha 24 hrs	$p=0.0561$, $F_{1,152}=3.70$	36		N/A
Estradiol				
Beta gonad > beta muscle	$p<0.0001$, $F_{1,152}=562.98$	36	0.00142	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,152}=453.92$	36	0.00146	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,152}=414.25$	36	0.00151	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,152}=302.96$	36	0.00155	Y
Stable gonad > stable muscle	$p<0.0001$, $F_{1,152}=302.94$	36	0.00160	Y
Beta gonad > beta brain	$p<0.0001$, $F_{1,152}=280.95$	36	0.00165	Y
Alpha gonad > alpha brain	$p<0.0001$, $F_{1,152}=195.92$	36	0.00171	Y
6 days gonad > 6 days brain	$p<0.0001$, $F_{1,152}=172.94$	36	0.00177	Y
24 hrs gonad > 24 hrs brain	$p<0.0001$, $F_{1,152}=153.11$	36	0.00183	Y
Stable gonad > stable brain	$p<0.0001$, $F_{1,152}=144.94$	36	0.00190	Y
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,152}=52.51$	36	0.00197	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,152}=52.42$	36	0.00205	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,152}=49.57$	36	0.00213	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,152}=28.83$	36	0.00223	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,152}=28.48$	36	0.00233	Y
Beta 6 days > beta 24 hrs	$p=0.0001$, $F_{1,152}=15.19$	36	0.00244	Y
Beta 6 days > alpha 6 days	$p=0.0002$, $F_{1,152}=14.27$	36	0.00256	Y
Beta gonad > alpha gonad	$p=0.0053$, $F_{1,152}=8.01$	36	0.00270	N
6 days gonad > stable gonad	$p=0.0100$, $F_{1,152}=6.81$	36	0.00285	N
Beta 6 days > beta stable	$p=0.0175$, $F_{1,152}=5.78$	36	0.00301	N
6 days gonad > 24 hrs gonad	$p=0.0361$, $F_{1,152}=4.48$	36	0.00320	N
Beta stable > alpha stable	$p=0.0620$, $F_{1,152}=3.54$	36		N/A
6 days brain > 24 hrs brain	$p=0.0694$ $F_{1,152}=3.35$	36		N/A

These linear contrasts compare alpha and beta females in stable groups and in groups undergoing sex change (24 hr and 6 day). P-values shown are uncorrected. The adjusted α value is based on the number of possible comparisons subjected to sequential Dunn-Sidak adjustments $\{1-(1-\alpha)^{1/k}\}$ where 'k' is the comparison number for the particular correlation being adjusted. To remain significant after adjustment, the uncorrected p-value must be less than the adjusted α value.

Table 3.3: Three-way linear contrasts comparing females.

			Stable Groups						24 h Groups						6 d Groups					
			α			β			α			β			α			β		
			B	G	M	B	G	M	B	G	M	B	G	M	B	G	M	B	G	M
Stable	α	B																		
		G	51.7 <0.0001																	
		M	10.46 0.002	112.6 <0.0001																
	β	B	0.266 0.607																	
		G		9.658 0.002		96.6 <0.0001														
		M			0.272 0.603	19.1 <0.0001	194.7 <0.0001													
24 h	α	B	0.217 0.642			0.986 0.322														
		G		6.698 0.011			0.073 0.787		89.0 <0.0001											
		M			1.625 0.204			0.588 0.444	15.4 0.0001	167.7 <0.0001										
	β	B	0.007 0.932			0.183 0.670			0.303 0.583											
		G		0.827 0.365			4.952 0.028			3.149 0.078		64.2 <0.0001								
		M			0.151 0.698			0.014 0.905			0.747 0.389	13.1 0.0004	135.3 <0.0001							
6d	α	B	0.501 0.480			0.051 0.822			1.344 0.248			0.390 0.533								
		G		2.168 0.143			2.526 0.114			1.442 0.232			0.348 0.556		56.6 <0.0001					
		M			2.521 0.115			1.136 0.288			0.073 0.787			1.339 0.249	28.7 <0.0001	176.4 <0.0001				
	β	B	2.325 0.130			1.075 0.301			4.009 0.047			2.065 0.153			0.565 0.453					
		G		28.95 <0.0001			4.879 0.029			5.362 0.022			20.234 <0.0001			14.426 0.0002		125.0 <0.0001		
		M			0.094 0.759			0.687 0.408			2.477 0.118			0.472 0.493			3.591 0.060	21.0 <0.0001	240.3 <0.0001	

Estradiol showed a significant 3-way interaction so we performed 3-way linear contrasts and sequential Dunn-Sidak adjustments of the p-value. Within each cell of the table, the F-value is shown on top, p-value on bottom, all F-values have df=1, 143. Yellow cells indicate that the contrast is significant after adjustments; blue cells indicate a marginal significance after adjustments; green cells indicate non-significant (but notable) difference after adjustments. Stable alpha = alpha female; stable beta = beta female; in 24 hrs and 6 days groups: alpha = sex-changing female; beta = beta female rising to alpha status; B = brain, G = gonad, M = muscle.

Table 3.4: Linear contrasts between stable males and females in sex changing groups.

Contrast	p-value, F ratio	k	Adjusted α	Significant after adjustment?
Testosterone				
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,149}=211.16$	36	0.00142	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,149}=178.87$	36	0.00146	Y
Stable muscle > 6 days muscle	$p<0.0001$, $F_{1,149}=171.90$	36	0.00151	Y
Stable brain > stable gonad	$p<0.0001$, $F_{1,149}=150.50$	36	0.00155	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,149}=149.05$	36	0.00160	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,149}=138.08$	36	0.00165	Y
Stable muscle > 24 hrs muscle	$p<0.0001$, $F_{1,149}=133.08$	36	0.00171	Y
Stable brain > 6 days brain	$p<0.0001$, $F_{1,149}=108.60$	36	0.00177	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,149}=108.31$	36	0.00183	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,149}=104.28$	36	0.00190	Y
Alpha stable > alpha 6 days	$p<0.0001$, $F_{1,149}=103.37$	36	0.00197	Y
Beta stable > beta 6 days	$p<0.0001$, $F_{1,149}=92.78$	36	0.00205	Y
Stable brain > 24 hrs brain	$p<0.0001$, $F_{1,149}=89.30$	36	0.00213	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,149}=88.27$	36	0.00223	Y
Beta gonad > beta muscle	$p<0.0001$, $F_{1,149}=73.48$	36	0.00233	Y
Alpha stable > alpha 24 hrs	$p<0.0001$, $F_{1,149}=69.79$	36	0.00244	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,149}=66.28$	36	0.00256	Y
Beta stable > beta 24 hrs	$p<0.0001$, $F_{1,149}=64.42$	36	0.00270	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,149}=37.95$	36	0.00285	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,149}=23.28$	36	0.00301	Y
Beta gonad > alpha gonad	$p=0.0009$, $F_{1,149}=11.42$	36	0.00320	Y
Stable gonad > stable muscle	$p=0.0357$, $F_{1,149}=4.49$	36	0.00341	N
6 days brain > 6 days gonad	$p=0.0364$, $F_{1,149}=4.46$	36	0.00366	N

Table 3.4: Linear contrasts between stable males and females in sex changing groups (continued).

11-Ketotestosterone				
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,152}=344.80$	36	0.00142	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,152}=283.61$	36	0.00146	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,152}=257.79$	36	0.00151	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,152}=249.65$	36	0.00155	Y
Alpha brain > alpha gonad	$p<0.0001$, $F_{1,152}=153.96$	36	0.00160	Y
6 days brain > 6 days gonad	$p<0.0001$, $F_{1,152}=140.27$	36	0.00165	Y
Beta brain > beta gonad	$p<0.0001$, $F_{1,152}=136.33$	36	0.00171	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,152}=93.62$	36	0.00177	Y
24 hrs brain > 24 hrs gonad	$p<0.0001$, $F_{1,152}=89.94$	36	0.00183	Y
Stable brain > stable gonad	$p<0.0001$, $F_{1,152}=66.78$	36	0.00190	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,152}=36.98$	36	0.00197	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,152}=36.91$	36	0.00205	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,152}=26.53$	36	0.00213	Y
6 days brain > stable brain	$p<0.0001$, $F_{1,152}=20.32$	36	0.00223	Y
Beta gonad > beta muscle	$p<0.0001$, $F_{1,152}=19.53$	36	0.00233	Y
24 hrs brain > stable brain	$p<0.0001$, $F_{1,152}=16.07$	36	0.00244	Y
Alpha brain > beta brain	$p=0.0008$, $F_{1,152}=11.69$	36	0.00256	Y
Stable muscle > 6 days muscle	$p=0.0051$, $F_{1,152}=8.08$	36	0.00270	N
Alpha 6 days > beta 6 days	$p=0.0072$, $F_{1,152}=7.43$	36	0.00285	N
Stable muscle > 24 hrs muscle	$p=0.0102$, $F_{1,152}=6.77$	36	0.00301	N
Alpha gonad > beta gonad	$p=0.0143$, $F_{1,152}=6.15$	36	0.00320	N
24 hrs gonad > stable gonad	$p=0.0287$, $F_{1,152}=4.88$	36	0.00341	N
Alpha stable > beta stable	$p=0.0339$, $F_{1,152}=4.58$	36	0.00366	N

Table 3.4: Linear contrasts between stable males and females in sex changing groups (continued).

Estradiol				
Beta gonad > beta muscle	$p<0.0001$, $F_{1,145}=419.19$	36	0.00142	Y
6 days gonad > 6 days muscle	$p<0.0001$, $F_{1,145}=364.47$	36	0.00146	Y
Alpha gonad > alpha muscle	$p<0.0001$, $F_{1,145}=334.49$	36	0.00151	Y
24 hrs gonad > 24 hrs muscle	$p<0.0001$, $F_{1,145}=266.55$	36	0.00155	Y
Beta gonad > beta brain	$p<0.0001$, $F_{1,145}=204.34$	36	0.00160	Y
6 days gonad > 6 days brain	$p<0.0001$, $F_{1,145}=152.16$	36	0.00165	Y
Stable gonad > stable muscle	$p<0.0001$, $F_{1,145}=140.24$	36	0.00171	Y
24 hrs gonad > 24 hrs brain	$p<0.0001$, $F_{1,145}=134.72$	36	0.00177	Y
6 day gonad > stable gonad	$p<0.0001$, $F_{1,145}=128.45$	36	0.00183	Y
Alpha 6 days > alpha stable	$p<0.0001$, $F_{1,145}=126.43$	36	0.00190	Y
Alpha 24 hrs > alpha stable	$p<0.0001$, $F_{1,145}=124.53$	36	0.00197	Y
Beta stable > alpha stable	$p<0.0001$, $F_{1,145}=122.70$	36	0.00205	Y
Alpha gonad > alpha brain	$p<0.0001$, $F_{1,145}=86.69$	36	0.00213	Y
Alpha brain > alpha muscle	$p<0.0001$, $F_{1,145}=84.36$	36	0.00223	Y
24 hrs gonad > stable gonad	$p<0.0001$, $F_{1,145}=80.01$	36	0.00233	Y
Stable brain > stable muscle	$p<0.0001$, $F_{1,145}=52.88$	36	0.00244	Y
6 days brain > 6 days muscle	$p<0.0001$, $F_{1,145}=43.61$	36	0.00256	Y
Beta gonad > alpha gonad	$p<0.0001$, $F_{1,145}=40.59$	36	0.00270	Y
Beta brain > beta muscle	$p<0.0001$, $F_{1,145}=38.16$	36	0.00285	Y
Beta muscle > alpha muscle	$p<0.0001$, $F_{1,145}=28.30$	36	0.00301	Y
24 hrs brain > 24 hrs muscle	$p<0.0001$, $F_{1,145}=25.06$	36	0.00320	Y

Table 3.4: Linear contrasts between stable males and females in sex changing groups (continued).

Stable gonad > stable brain	$p<0.0001$, $F_{1,145}=20.34$	36	0.00341	Y
Beta 6 days > beta stable	$p<0.0001$, $F_{1,145}=16.20$	36	0.00366	Y
6 day muscle > stable muscle	$p=0.0001$, $F_{1,145}=15.41$	36	0.00394	Y
Beta 6 days > beta 24 hrs	$p=0.0004$, $F_{1,145}=13.36$	36	0.00427	Y
24 hrs muscle > stable muscle	$p=0.0005$, $F_{1,145}=12.56$	36	0.00465	Y
Beta 6 days > alpha 6 days	$p=0.0005$, $F_{1,145}=12.55$	36	0.00512	Y
6 day brain > stable brain	$p=0.0016$, $F_{1,145}=10.36$	36	0.00568	Y
Beta brain > alpha brain	$p=0.0353$, $F_{1,145}=4.51$	36	0.00639	N
6 day gonad > 24 hrs gonad	$p=0.0491$, $F_{1,145}=3.94$	36	0.00730	N

List of all linear contrasts that are significant or show a trend towards significant values when comparing hormone levels of alpha and beta females at 24 hr and 6 days after male removal with those respectively of males and alpha females in stable groups. In this table, the word "alpha stable" refers to males because they have alpha status, the word "beta stable" refers to alpha in stable groups because they are beta to the male. P-values shown are uncorrected. The adjusted α value is based on the number of possible comparisons subjected to sequential Dunn-Sidak adjustments $\{1-(1-\alpha)^{1/k}\}$ where 'k' is the comparison number for the particular correlation being adjusted. To remain significant after adjustment, the uncorrected p-value must be less than the adjusted α value.

Table 3.5: Three-way linear contrasts comparing males and females.

			Stable Groups						24 h Groups						6 d Groups					
			α			β			α			β			α			β		
			B	G	M	B	G	M	B	G	M	B	G	M	B	G	M	B	G	M
Stable	α	B																		
		G	0.308 0.580																	
		M	53.4 <0.0001	45.6 <0.0001																
	β	B	6.216 0.014																	
		G		99.04 <0.0001		45.5 <0.0001														
		M			45.59 <0.0001	9.21 0.003	99.1 <0.0001													
24 h	α	B	4.183 0.043			0.191 0.663														
		G		128.4 <0.0001			5.893 0.016		78.3 <0.0001											
		M			28.90 <0.0001			1.430 0.234	13.6 0.0003	147.5 <0.0001										
	β	B	6.633 0.011			0.006 0.936			0.267 0.606											
		G		117.2 <0.0001			0.728 0.395			2.770 0.098		56.5 <0.0001								
		M			38.5 <0.0001			0.133 0.716			0.657 0.419	11.5 0.0009	119.0 <0.0001							
6 d	α	B	9.579 0.002			0.441 0.508			1.182 0.279			0.343 0.559								
		G		122.21 <0.0001			1.907 0.169			1.269 0.262			0.306 0.581		49.8 <0.0001					
		M			27.70 <0.0001			2.218 0.139			0.064 0.800			1.178 0.280	25.2 <0.0001	155.2 <0.0001				
	β	B	16.25 <0.0001			2.045 0.395			3.527 0.062			1.817 0.180			0.498 0.482					
		G		219.34 <0.0001			25.47 <0.0001			4.717 0.031			17.80 <0.0001			12.693 0.0005		109.9 <0.0001		
		M			49.56 <0.0001			0.084 0.774			2.180 0.142			0.416 0.520			3.159 0.078	18.5 <0.0001	211.4 <0.0001	

Estradiol showed a significant 3-way interaction so we performed 3-way linear contrasts and sequential Dunn-Sidak adjustments of the p-value. Within each cell of the table, the F-value is shown on top, p-value on bottom, all F-values have df = 1, 145; yellow cells indicate that the contrast is significant after adjustments; blue cells indicate a marginal significance after adjustments; green cells indicate non-significant (but notable) difference after adjustments. Stable alpha = male; stable beta = alpha female; in 24 hrs and 6 days groups, alpha = sex-changing alpha female; beta = beta female rising to alpha status; B = brain, G = gonad, M = muscle.

Table 3.6: Correlations between hormones in different tissues.

	p-value, F ratio	R^2	slope b
Alpha stable			
Gonad KT by Gonad T	$p<0.0001$, $F_{1,8}=200.62$	0.962	0.0126
Muscle KT by Muscle T	$p<0.0001$, $F_{1,8}=87.41$	0.916	0.005
Gonad E2 by Muscle E2	$p=0.0422$, $F_{1,7}=6.15$	0.468	-14.643
Beta stable			
Gonad KT by Gonad T	$p<0.0001$, $F_{1,8}=76.97$	0.906	0.0103
Muscle KT by Muscle T	$p<0.0001$, $F_{1,8}=305.35$	0.974,	0.0053
Gamma stable			
Gonad T by Brain T	$p=0.0444$, $F_{1,8}=5.67$	0.415	0.0881
Gonad KT by Brain T	$p=0.0105$, $F_{1,8}=11.06$	0.580	0.0007
Gonad KT by Gonad T	$p<0.0001$, $F_{1,8}=184.83$	0.959	0.0067
Muscle KT by Muscle T	$p=0.0028$, $F_{1,6}=23.54$	0.797	0.0061
Male stable			
Muscle KT by Muscle T	$p<0.0001$, $F_{1,8}=95.24$	0.923	0.0059
Alpha 24 hrs			
Muscle KT by Gonad KT	$p=0.0018$, $F_{1,6}=28.38$	0.825	0.132
Beta 24 hrs			
Brain KT by Brain T	$p<0.0001$, $F_{1,7}=152.61$	0.956	0.0615
Brain E2 by Gonad E2	$p=0.0255$, $F_{1,7}=7.80$	0.533	0.0365
Gonad KT by Gonad T	$p<0.0001$, $F_{1,6}=2976.10$	0.998	0.0142
Muscle KT by Muscle T	$p<0.0001$, $F_{1,7}=82.54$	0.922	0.0107
Alpha 6 days			
Gonad E2 by Brain E2	$p=0.0130$, $F_{1,4}=18.22$	0.820	10.405
Gonad E2 by Gonad KT	$p=0.0138$, $F_{1,6}=11.83$	0.663	-85.003

Table 3.6: Correlations between hormones in different tissues (continued).

Brain E2 by Gonad KT	$p=0.0117$, $F_{1,6}=12.80$	0.681	-6.400
Muscle T by Brain E2	$p=0.0056$, $F_{1,6}=17.80$	0.748	0.238
Muscle T by Gonad KT	$p=0.0132$, $F_{1,8}=10.04$	0.556	-1.589
Beta 6 days			
Brain KT by Brain T	$p=0.0003$, $F_{1,8}=38.44$	0.828	0.086
Gonad KT by Gonad T	$p=0.0241$, $F_{1,8}=7.70$	0.490	0.012
Muscle T by Gonad KT	$p=0.0211$, $F_{1,8}=8.18$	0.506	54.917
Muscle KT by Gonad KT	$p=0.0053$, $F_{1,8}=14.40$	0.643	0.383
Muscle KT by Muscle T	$p<0.0001$, $F_{1,8}=203.80$	0.962	0.0061
All females together, all treatments			
Gonad KT by Brain KT	$p=0.0544$, $F_{1,65}=3.85$	0.058	0.0728
Gonad KT by Gonad T	$p<0.001$, $F_{1,65}=22.73$	0.259	0.0045
Gonad KT by Muscle T	$p=0.0229$, $F_{1,65}=5.43$	0.078	-0.0057
Muscle KT by Muscle T	$p<0.001$, $F_{1,66}=411.85$	0.862	0.0056
All fish together			
Gonad KT by Brain KT	$p=0.024$, $F_{1,72}=5.31$	0.069	0.0745
Gonad KT by Gonad T	$p<0.0001$, $F_{1,75}=24.66$	0.247	0.0044
Muscle KT by Muscle T	$p<0.0001$, $F_{1,75}=456.25$	0.857	0.0058
Muscle E2 by Brain E2	$p=0.0024$, $F_{1,72}=9.92$	0.120	0.197

Table 3.7: Correlations between hormones and behavior.

	p-value, F ratio	R^2	slope b
Male, stable 7 days			
Gonad E2 by total approaches + aggression	p=0.0223, $F_{1,8}$=7.99	0.500	-2.818
Gonad E2 by total approaches	p=0.0305, $F_{1,8}$=6.88	0.463	-5.488
Gonad E2 by total displacements	p=0.0139, $F_{1,8}$=9.82	0.551	-6.535
Alpha Female, Sex change 24 hrs			
Gonad KT by approaches 1 hr after male removal	p=0.0111, $F_{1,6}$=13.13	0.686	1.014
Muscle KT by approaches 1 hr after male removal	p=0.0461, $F_{1,7}$=5.86	0.456	0.121
Gonad KT by total approaches after male removal	p=0.0025, $F_{1,6}$=24.96	0.810	1.345
Muscle KT by total approaches after male removal	p=0.0044, $F_{1,7}$=17.11	0.710	0.187
Gonad KT by total approaches before & after male removal	p=0.0022, $F_{1,6}$=25.88	0.812	1.204
Muscle KT by total approaches before & after male removal	p<0.0001, $F_{1,7}$=108.35	0.939	0.191
Alpha Female, Sex change 6 days			
Muscle E2 by Total approaches + aggression 1 hr after male removal	p=0.0035, $F_{1,8}$=16.68	0.676	0.534
Muscle E2 by attacks 1 hr after male removal	p=0.0035, $F_{1,8}$=16.76	0.677	1.580
Muscle E2 by approaches 1 hr after male removal	p=0.0144, $F_{1,8}$=9.70	0.548	2.268
Muscle E2 by displacements 1 hr after male removal	p=0.0043, $F_{1,8}$=15.48	0.659	1.109
Muscle KT by total displacements after male removal	p=0.042, $F_{1,8}$=5.84	0.422	0.028
Gonad KT by tot approaches before male removal	p=0.0055, $F_{1,8}$=14.21	0.640	-1.659
Brain E2 by attacks 1 day after male removal	p=0.0246, $F_{1,6}$=8.90	0.597	3.749
Beta Female, Sex change 24 hours			
Brain T by Total displaced 1 day After male removal	p=0.0178, $F_{1,7}$=9.49	0.575	44.243
Brain KT by Total displaced 1 day After male removal	p=0.0065, $F_{1,7}$=14.65	0.677	3.016

Table 3.7: Correlations between hormones and behavior (continued).

Beta Female, Sex change 6 days			
Brain T by Total displaced 5 day PM After male removal	p=0.0114, $F_{1,8}$=10.67	0.572	-33.835
Brain KT by Total displaced 5 day PM After male removal	p=0.0134, $F_{1,8}$=9.98	0.555	-3.154
Gonad KT by Total displaced After male removal	p=0.0026, $F_{1,8}$=18.49	0.698	0.386
Gonad T by Total approaches + aggression 5 day PM After male removal	p=0.0463, $F_{1,8}$=5.55	0.409	-11.728
Gonad E2 by Total approaches + aggression Before & After male removal	p=0.0103, $F_{1,6}$=13.54	0.693	540.599
Gonad E2 by displacements Before male removal	p=0.0174, $F_{1,6}$=10.57	0.638	495.947
Gonad KT by approaches 5 day PM After male removal	p=0.0470, $F_{1,8}$=5.50	0.408	-0.443
Gonad T by displacements 5 day PM After male removal	p=0.0463, $F_{1,8}$=5.55	0.409	-23.456
All fish together			
Brain T by Total approaches + aggression 5 day AM After male removal	p=0.0115, $F_{1,16}$=8.13	0.337	0.030
Brain T by attacks 5 day AM After male removal	p=0.0003, $F_{1,16}$=21.09	0.569	0.014
Brain T by displacements 5 day AM After male removal	p=0.0069, $F_{1,16}$=9.60	0.375	0.015
Brain KT by Total approaches + aggression Before & After male removal	p=0.0012, $F_{1,16}$=12.40	0.267	0.263
Brain KT by attacks Before & After male removal	p=0.0012, $F_{1,16}$=12.54	0.269	0.080
Brain KT by displacements Before & After male removal	p=0.0010, $F_{1,16}$=12.99	0.276	0.135
Gonad T by Total approaches + aggression 5 day AM After male removal	p=0.059, $F_{1,16}$=4.06	0.184	- 0.051
Gonad T by attacks 5 day AM After male removal	p=0.0510, $F_{1,16}$=4.37	0.195	- 0.019

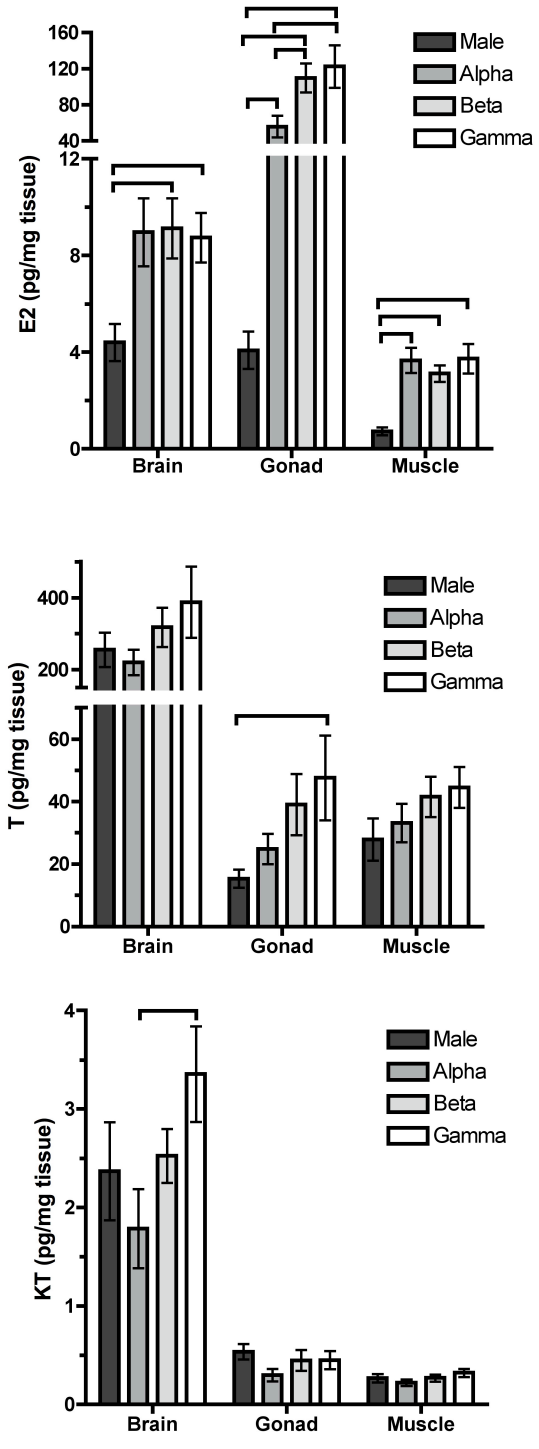


Figure 3.1: Concentrations of estradiol (E2), testosterone (T) and 11-ketotestosterone (KT) in stable groups. Lines between bars represent significant differences.

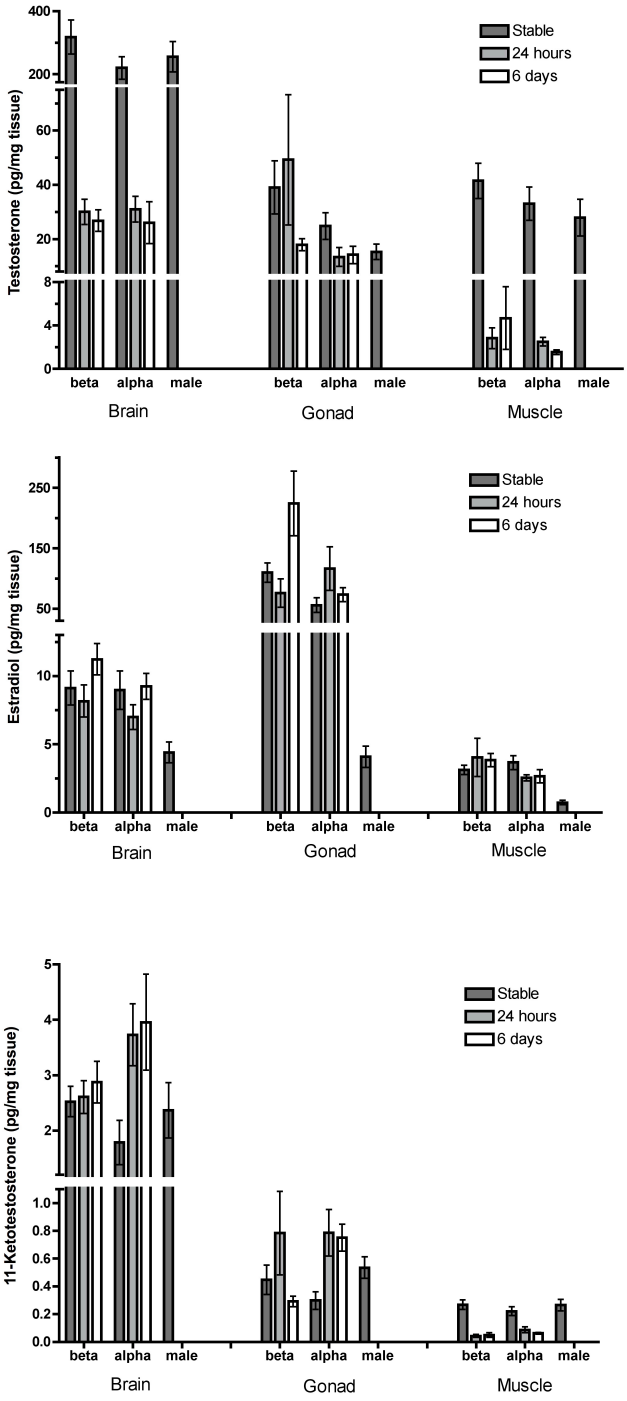


Figure 3.2: Concentrations of testosterone, estradiol and 11-ketotestosterone in stable and sex changing groups. The legends "24 hours" and "6 days" refer to time after male removal.

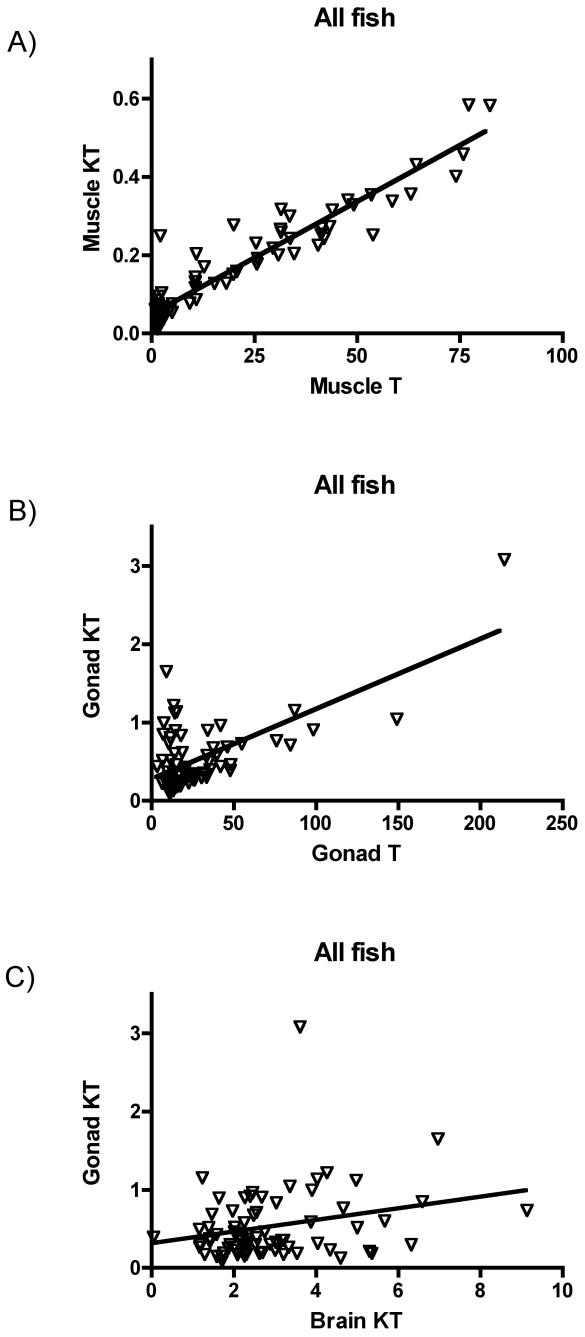


Figure 3.3: Correlations between concentration of KT and T in the muscle (A), of KT and T in the gonad (B), and of KT in the gonad versus KT in the brain (C) across all fish from stable and sex changing groups. All correlations are significant ($p<0.05$).

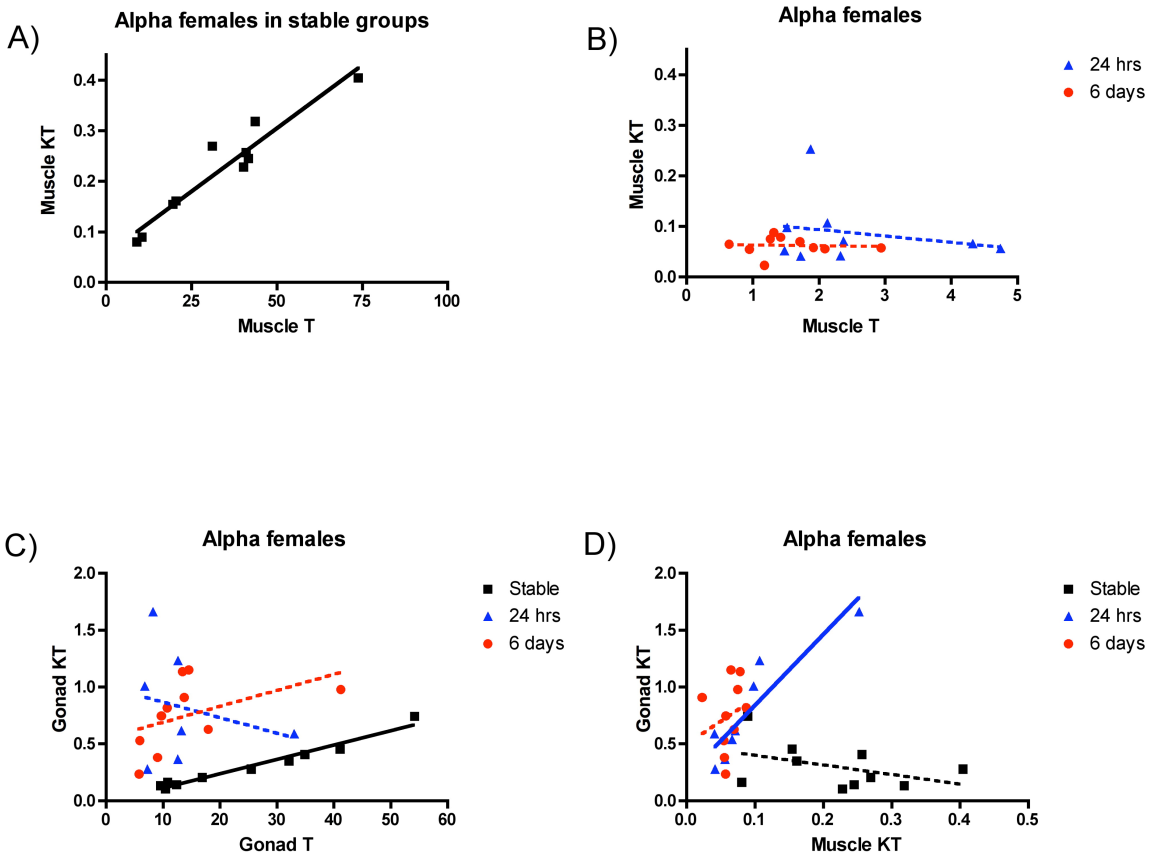


Figure 3.4: Correlations between concentration of KT and T in the muscle in stable groups (A) and in sex changing groups (B), and between concentration of KT versus T in the gonad (C), and between concentration of KT in the muscle versus the gonad (D). All the values refer to alpha females only. A solid line means that the associated p value is significant ($p<0.05$) while dotted lines are associated with non-significant correlations.

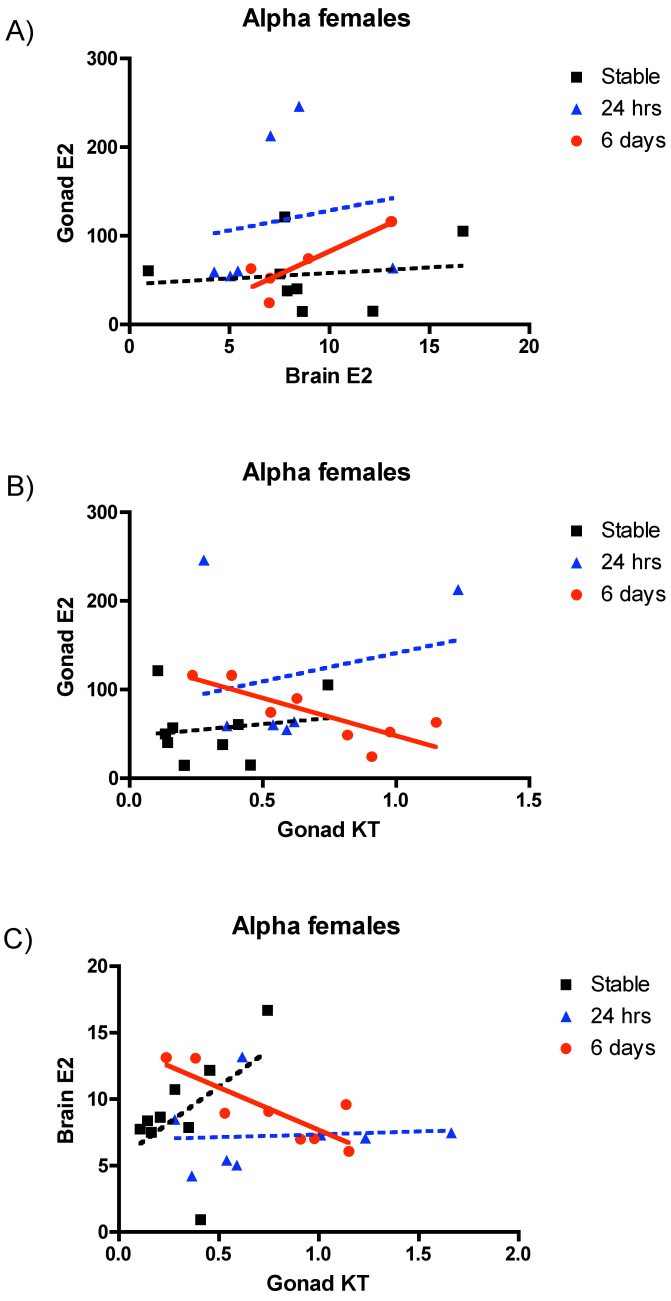


Figure 3.5: Correlation between concentration of E2 in the brain versus the gonad (A), between concentration of KT versus E2 in the gonad (B), and between concentration of KT in the gonad versus E2 in the brain (C). All the values refer to alpha females only. A solid line means that the associated p value is significant ($p<0.05$) while dotted lines refer to non-significant correlations.

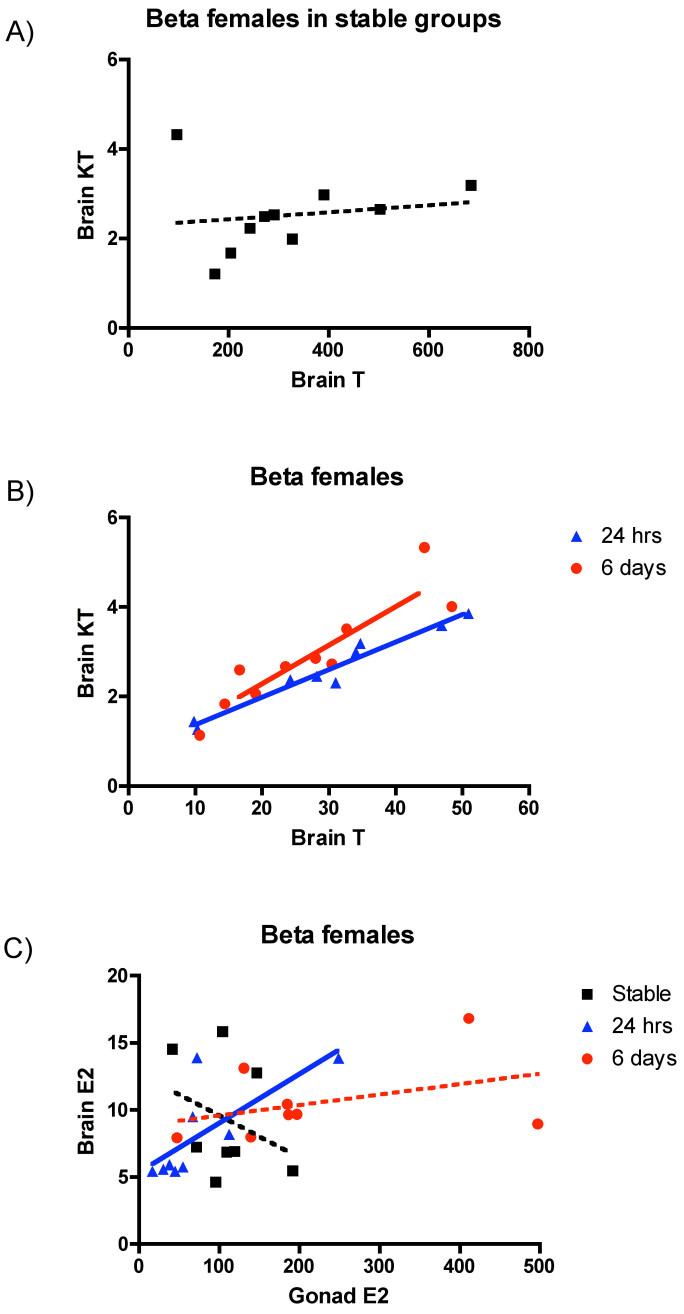


Figure 3.6: Correlation between concentration of T versus KT in the brain in stable groups (A), and in sex changing groups (B), and between concentrations of E2 in the gonad versus the brain (C). All the values refer to beta females only. A solid line means that the associated p value is significant ($p<0.05$) while dotted lines refer to non-significant correlations.

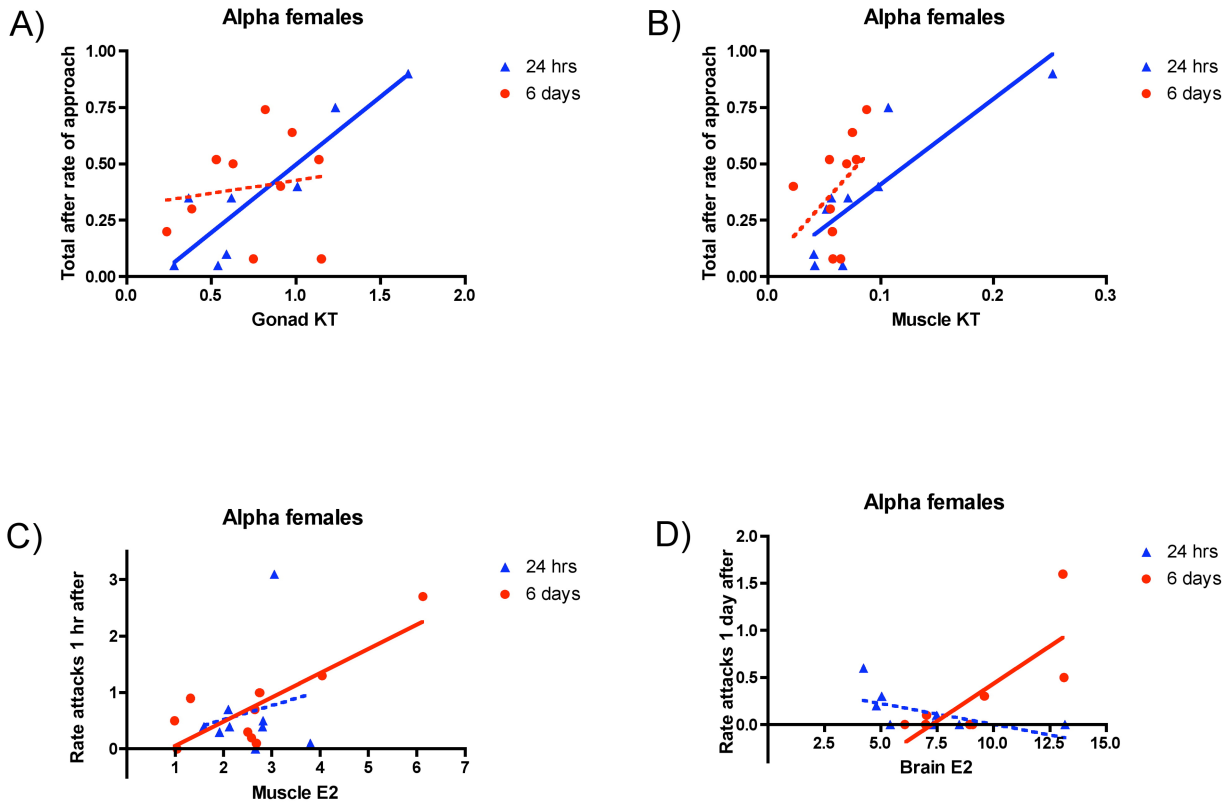


Figure 3.7: Correlation between the total rate of approaches performed after male removal and concentration of KT in the gonad (A), and in the muscle (B); between the rate of attacks performed an hour after male removal and concentration of E2 in the muscle (C); and between the rate of attacks performed the day after male removal and concentration of E2 in the brain (D). The values refer to alpha females in sex changing groups collected 24 hours and 6 days after male removal. A solid line means that the associated p value is significant ($p<0.05$) while dotted lines refer to non-significant correlations.

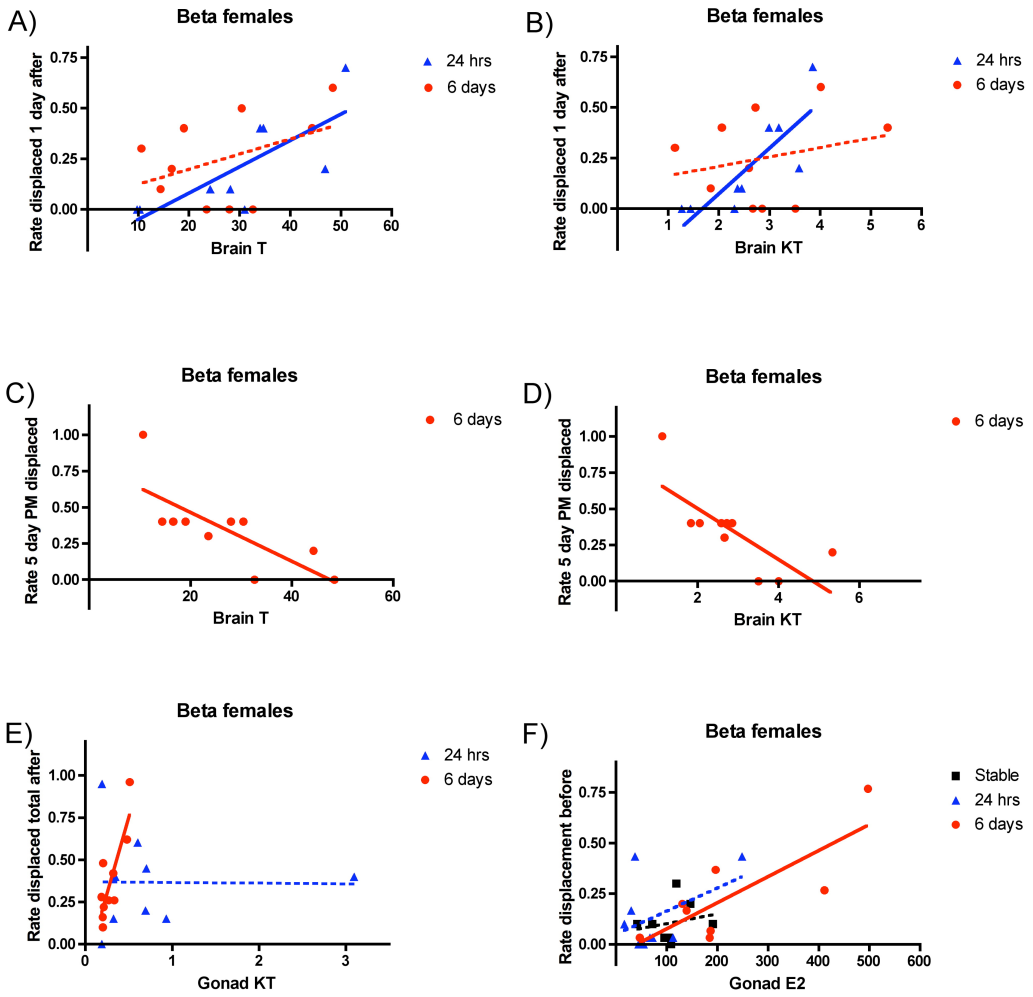


Figure 3.8: Correlation between the rate of displacements received 1 day after male removal and brain concentration of T (A), and KT (B); between the rate of displacements received in the afternoon 5 days after male removal and brain concentration of T (C) and KT (D); between the total rate of displacements received after male removal and concentration of KT in the gonad (E); and between the rate of displacements given before male removal and concentration of E2 in the gonad (F). The values are from beta females in sex changing groups collected 24 hours and 6 days after male removal. A solid line means that the associated p value is significant ($p<0.05$) while dotted lines refer to non-significant correlations.

CHAPTER 4

SEROTONIN, SOCIAL STATUS AND SEX CHANGE IN THE BLUEBANDED GOBY *LYTHRYPNUS DALLI*

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Contributions:

R. E. Carpenter and C. H. Summers assisted in the HPLC analysis, R. L. Earley assisted in the statistical analysis and the editing of the manuscript.

Abstract

In a variety of vertebrates, highly aggressive individuals tend to have high social status and low serotonergic function. In the sex changing fish *Lythrypnus dalli*, serotonin (5-HT) may be involved as a mediator between the social environment and the reproductive system because social status is a critical cue in regulating sex change. Subordination inhibits sex change in *L. dalli*, and it is associated with higher serotonergic activity in other species. We tested the hypothesis that high serotonergic activity has an inhibitory effect on sex change. In a social situation permissive to sex change, we administered to the dominant female implants containing the serotonin precursor 5-hydroxytryptophan (5-HTP). In a social situation not conducive to sex change, we administered either the serotonin synthesis inhibitor p-chlorophenylalanine (PCPA) or the 5-HT_{1A} receptor antagonist p-MPPI. After three weeks we used HPLC to measure brain

levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). We also performed PCPA, p-MPPI and fluoxetine injections in size-matched pairs of females to assess its effect on dominance status. Males and newly sex changed fish showed a trend for higher levels of 5-HIAA and 5-HT/5-HIAA ratio than females. The different implants treatments did not affect the probability of sex change. Interestingly, this species does not seem to fit the pattern seen in other vertebrates where dominant individuals have lower serotonergic activity than subordinates.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that mediates a wide variety of behavioral functions through its action in both the central and peripheral nervous systems. Aggressive behavior has been associated with serotonergic function (Adams et al., 1996; Edwards and Kravitz, 1997; Larson and Summers, 2001) in a variety of taxa ranging from lobsters (Kravitz, 2000) to macaques (Howell et al., 2007). In vertebrates, high serotonergic function is often associated with low levels of aggression and/or subordinate status. High serotonergic function has been induced in various studies by using 5-HT receptor agonists or selective serotonin reuptake inhibitors (SSRIs), which retains 5-HT for a longer period in the synapse. For example, in the lizard *Anolis carolinensis*, chronic SSRI administration to dominant males reversed their social status in a pair, and decreased their production of aggressive behavior (Deckel, 1996; Larson and Summers, 2001). In male song sparrows, administering the SSRI fluoxetine or a 5-HT_{1A} receptor agonist decreased aggressive behavior (Sperry et al., 2003). Bluehead wrasses *Thalassoma bifasciatum* treated with fluoxetine, either chronically for 14 days or with one single injection, decreased their aggression towards an intruder (Perreault et al., 2003). Higher turnover of serotonin is commonly quantified by measuring the accumulation of

its metabolite 5-hydroxyindoleacetic acid (5-HIAA). In fishes, brain levels of 5-HIAA were higher in subordinate than dominant individuals in both rainbow trout *Oncorhyncus mykiss* (Winberg and Lepage, 1998), and juvenile arctic charr *Salvelinus alpinus* (Winberg et al., 1991). This was shown to be the product of social interactions rather than intrinsic individual differences (Winberg et al., 1992).

Similarly, there is evidence suggesting that low serotonergic function is associated with heightened aggression and/or social dominance. Lines of mice selected for high aggressiveness had lower serotonin levels in the prefrontal cortex than lines selected for low aggressiveness (Caramaschi et al., 2007). In stickleback *Gasterosteus aculeatus*, there was a negative correlation between 5-HT and levels of aggression against an intruder (Bell et al., 2007). A single injection of the serotonin synthesis inhibitor, p-clorophenilalanine (PCPA), increased aggression towards a mirror in the firemouth cichlid *Cichlasoma meeki* (Adams et al., 1996).

The relationship between 5-HT and aggression is, however, quite complex and it is not clear whether they are causally related. For instance, 5-HT activity increases in both dominant and subordinate animals during a fight so its inhibitory action on aggression might be tightly linked to specific brain regions or might depend on the stage of aggressive contests (Summers et al., 2005; Summers and Winberg, 2006). Also, studies on mice have shown that the anti-aggressive effect of 5-HT_{1A} and 5-HT_{1B} agonists are actually due to their action on the pre-synaptic autoreceptors and therefore associated with a reduction rather than increase of 5-HT neurotransmission (de Boer and Koolhaas, 2005).

In addition to the relationship between the serotonergic system and aggressive behavior, there also is clear evidence for a link with reproductive function (Fabre-Nys, 1998; Hull et al., 2004). In rodents, 5-HT has an inhibitory effect on reproductive behavior (Malmnas and

Meyerson, 1971; Verma et al., 1989); and seems to be exerting this effect through the 5-HT_{1A} receptors (Kishitake and Yamanouchi, 2003, 2004). In fish, 5-HT can have both inhibitory and stimulatory actions on the reproductive system. For example, the yellow snapper *Lutjanus argentiventris* exhibits changes in telencephalic 5-HT and 5-HIAA levels over the course of the reproductive cycle with lowest levels during the spawning season (Hernandez-Rauda and Aldegunde, 2002). In female rainbow trout, brain 5-HIAA levels were lowest during vitellogenesis and peaked during the periovulatory period (Saligaut et al., 1992). Serotonin can facilitate the release of gonadotropins from the pituitary in the Atlantic croaker *Micropogonias undulates* (Khan and Thomas, 1992), sailfin molly *Poecilia latipinna* (Groves and Batten, 1986) and goldfish *Carassius auratus* (Somoza and Peter, 1991; Somoza et al., 1988). Also, in tilapia *Oreochromis mossambicus*, treatment with estrogens or PCPA during development decreases 5-HT brain levels and produces a female biased sex ratio (Tsai et al., 2000). This suggests that the feminizing effect of estrogen during sex differentiation might be mediated through 5-HT action.

Many species of fish are capable of sex change and, in some, the social environment serves as the cue that controls the switch between sexes (Fishelson, 1970; Godwin et al., 2003; Robertson, 1972; Rodgers et al., 2007; Shapiro, 1979; Warner and Swearer, 1991). In many protogynous species, removing the male induces sex change in the dominant female. Because the serotonergic system is involved in the modulation of both aggressive and reproductive behavior, changes in serotonergic function could be responsible for translating a change in social context (e.g., dominance status and/or aggression) into physiological and neurobiological modifications that both initiate and maintain sex reversal. Some experimental evidence for a role of serotonin in the inhibition of sex change comes from the protogynous saddleback wrasse *Thalassoma duperrey* (Larson et al., 2003a; Larson et al., 2003b). The SSRI sertraline inhibited gonadal sex

change in a female in the presence of a smaller female, a situation usually conducive to sex change in this species (Larson et al., 2003a).

To test the role of serotonin in the regulation of sex change, we used the bluebanded goby *Lythrypnus dalli*. At the functional level, this species is a sequential hermaphrodite because individuals exhibit only one behavioral sex at a time (Reavis and Grober, 1999; St Mary, 1994) but they are capable of bidirectional sex change and social status appears to play a critical role in regulating sexual transformations (Rodgers et al., 2007). Because elevated serotonergic activity typically is associated with subordinate status in vertebrates, and because 5-HT can affect reproductive function, we hypothesized that female *L. dalli*, being the subordinate animals, would have higher serotonergic activity than males, and that this higher serotonergic activity inhibits them from changing sex. To test this hypothesis, we used both pharmacological and social manipulations of the sex-changing individual (see Table 1 for summary of treatments). We induced high 5-HT levels in a dominant female and predicted that this would inhibit sex change in a social environment permissive to sex change. We also induced low serotonergic function and predicted that this would induce sex change in a non-permissive environment. As a result of these experiments, we established that the general rule for serotonin and status in vertebrates with dominants having lower serotonergic activity than subordinates does not seem to apply to the bluebanded goby. Therefore, we also tested fish in pairs to determine whether acute 5-HT manipulations can affect social status or aggression levels in *L. dalli*, and whether differences in brain serotonergic activity exist between dominant and subordinate individuals. Although the focus of the work was on 5-HT and 5-HIAA, we also measured brain levels of norepinephrine (NE), dopamine (DA) and its metabolite because of their possible effect on social status (Korzan and Summers, 2007; Winberg and Nilsson, 1992) and sex change (Larson et al., 2003a).

Materials and Methods

Study organism

The bluebanded goby is a small benthic fish (20-45 mm adult standard length) that inhabits rocky reefs along Southern California and Baja California, Mexico (Wiley, 1976). *Lythrypnus dalli* establishes social hierarchies with a dominant male that defends a nest, and spawns with multiple females within a given season (Behrents, 1983). Fish used in our study were captured off the coast of Santa Catalina Island, CA (California Department of Fish & Game permit # 803036-03 to VL) during summer 2006 and 2007. The fish were held in a large 197 l (60 x 94 x 35 cm) holding tank until further processing; the holding tank was supplied continuously with seawater. Fish were housed either in indoor seawater tables at the Wrigley Institute of Environmental Studies on Catalina Island (implant experiment and fluoxetine/p-MPPI injection experiment) exposed to the natural summer light cycle through large windows or in aquaria at Georgia State University (PCPA injection experiment) on a 12:12 light/dark photoperiod. They were held at a temperature of 18-20°C. The fish were fed brine shrimp twice daily. When setting up experimental groups in tanks, the sex of the fish was assessed by examining the genital papilla shape because males typically have thin and pointy papillae, while females have round, short and wide papillae (St. Mary, 1993; Wiley, 1976). At the start and end of each experiment, we took genital papilla pictures to measure the papilla length-to-width ratio. This ratio is a good indicator of papilla shape and therefore of sex, with female ratios being close to 1 and male ratios ≥ 1.4 (Carlisle et al., 2000). At the end of the experiment the gonads were removed and photographed to confirm the final sex of the animal. Male *L. dalli* always possess an accessory gonadal structure (AGS), filled with mucous and/or sperm (Drilling and Grober, 2005). The presence of the AGS is a good indicator of functional sex in this species because individuals with

rounded, female-like papilla might have some testicular tissue but lack AGS and they do not spawn as a male. The research conducted herein was approved by the Georgia State University IACUC protocol No. A06004 (0708) and University of Southern California IACUC protocol No. 10262.

Implant experiment

Social groups of one large male and three females were established in individual tanks. Males were at least 3 mm larger than the largest female in the tank to ensure their dominance. In each tank, the focal female was at least 3 mm larger than the other two females in her tank, to ensure that she would become the dominant female. Standard length ranges and averages (mean ± standard error) for the fish composing the social groups were: male 32.4-43.1 mm (36.8±0.6); largest female 29.2-33.1 mm (30.8±0.2); medium size female 25.5-28.9 mm (26.8±0.2); smallest female 23.2-26.1 mm (24.9±0.1). This type of hierarchy has been very successful at predicting sex change in the dominant female after male removal (Lorenzi et al., 2006; Rodgers et al., 2007). Five days after social group establishment, the dominant females were given one of the following treatments (all drugs were purchased from Sigma-Aldrich Co.): the serotonin precursor 5-hydroxytryptophan (5-HTP), the serotonin synthesis inhibitor para-chlorophenylalanine (PCPA), or the 5-HT_{1A} receptor antagonist 4-Iodo-N-[2-[-4-(methoxyphenyl)-1-piperazinyl] ethyl]-N-2-pyridinylbenzamide hydrochloride (p-MPPI). The implants were made by melting beeswax and mixing it with the drug in a ratio of 1 mg of drug to 5 mg of wax (Larson et al., 2003a), aspirating it into a thin silastic tubing (0.64 mm ID x 1.19 mm OD), peeling the tubing off, and cutting it into single 3 mm long implants. Control groups received implants made of beeswax only without any drug. The implants were inserted in the body cavity of the dominant female by anesthetizing with tricaine methanesulfonate (MS222) at a dosage of 150 mg/l,

making a small incision, and closing the wound with veterinary glue (Nexaband® S/C). When the dominant female received either the 5-HTP implant or the control wax implant, the male was removed from the tank three days after the surgery to create a social situation conducive to sex change. When the dominant female received either the PCPA implant or the p-MPPI implant, we left the male in the tank to produce an inhibitory social environment; on the third day after the surgery, we put the hand net in these tanks to simulate the same disturbance to the fish as in the male removal treatments.

The fish were observed every day for 10 minutes and scored for aggressive acts performed and received. The aggressive behaviors collected were: approaches defined as movement towards another fish within 2 body lengths of distance; displacements defined as approaches that end with the subordinate fish moving away; attacks defined as very quick and aggressive displacements; threat displays defined as aggressive display consisting of raising the dorsal fin and opening the opercula when very close to another fish. We also recorded jerk swims: a typical courtship behavior that males or sex changing fish perform towards the females or around the nest (Behrents, 1983; Reavis and Grober, 1999). Three weeks after male removal, animals were quickly moved with a hand-net from the tank into a beaker and killed via exposure to excess MS222 (1 g/l), the fish were decapitated, and their brains were rapidly removed, weighed fresh (± 0.1 mg), fast frozen on dry ice, and stored at - 80 °C until processing. Animals that no longer carried an implant in their body cavity were excluded from the analysis because they most likely lost it immediately or shortly after the surgery. The final sample size for each treatment is provided in Table 2. We also collected the brains of 6 of the males immediately after removing them from the experimental tanks and 10 untreated non-focal females at the end of the experiment.

Injection experiments

We established pairs of size-matched females (standard length differences were $\leq$ 0.1 mm). After measurement, they were held singly in an isolation tank for 2 days to reduce the effect of previous social experience. On the third day, both fish were anaesthetized with MS222 and received an i.p. injection of a serotonergic compound. Within each pair, one fish received the drug in 0.9% saline solution (vehicle), and the other one received the same volume of vehicle. Ten pairs received PCPA (Sigma-Aldrich Co.) at a dose of 0.2 mg/g body mass (Winberg and Nilsson, 1993b), ten received fluoxetine (Xeredien, Valeas S.p.A.) at a dose of 10 μg/g body mass (Perreault et al., 2003) and ten received p-MPPI (Sigma-Aldrich Co.) at a dose of 5 μg/g body mass. As there were no studies available on fish for p-MPPI, this dose was based on the range of concentrations that has a behavioral effect in rodents (Harrison et al., 2001; Markou et al., 2005). One fish in the p-MPPI treatment died so the final sample size in this treatment was 9 pairs. The fish were paired in an unfamiliar tank immediately after the PCPA injection or one hour after the fluoxetine or p-MPPI injection. Ten minute behavioral observations were performed immediately after pairing the fish, 3 hours later, and then once a day for the following five days. On the fifth day after the injection, the fish were sacrificed and brains were harvested as described in the implant experiment. In chicks (Buchanan et al., 1994), brain levels of 5-HT and 5-HIAA were still lower 5 days after a single i.p. injection of PCPA. We took pictures of the genital papillae and the gonads to determine final sexual phenotype. Brain monoamine levels were quantified only for the fish that received the PCPA treatment and their saline injected opponent (see below). Two samples from the HPLC analysis were unusable so we have brain measurements for N=18 animals (both the missing ones were saline injected and achieved dominant status).

Analysis of monoamines

Serotonin (5-HT), its metabolite 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE), dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using high performance liquid chromatography (HPLC) with electrochemical detection (Emerson et al., 2000; Renner and Luine, 1984, 1986). The ratio of metabolite to transmitter 5-HIAA/5-HT is used as a measure of serotonergic activity. The brain samples were sonicated in 100 µl of sodium acetate buffer (pH 5.0) containing 5 × 10- 8 M DHBA (9.4 pg/µl dihydroxybenzylamine; internal standard). Each sample was frozen, then thawed and centrifuged at 15000×g for 2 min. The supernatant was removed. We added 5 µl of ascorbic acid oxidase to each sample, and injected 45 µl of sample into a chromatographic system (Waters Associates, Milford, MA) and analyzed electrochemically with an LC-4B potentiostat (Bioanalytical Systems, West Lafayette, IN). The electrode potential was set at + 0.6 V with respect to an Ag/AgCl reference electrode. The mobile phase (pH=2.75, flow rate 1.8 ml min-1) consisted of 900 ml of deionized water, 250 mg NaOH, 18.63 mg Na_2EDTA, 21 g citric acid, 0.85 ml TEA, 105 mg sodium octane sulfonic acid (SOS) and 25 ml acetonitrile.

Statistical analysis

Analysis of variance (ANOVA) was used in the implant experiment, to compare levels of neurotransmitters between fish that received different drugs when normality (Shapiro Wilk test) and homogeneity of variance were achieved (Levene's test). A Wilcoxon test and the Kruskal Wallis test were used when these criteria were not met. Variance in levels of 5-HIAA in the implant experiment was not distributed homogenously so Kruskal Wallis was used to compare treatments. Wilcoxon was used in injection study to compare DA and NE levels. Tukey Kramer HSD was used as post hoc test following significant ANOVA results while Dunn's multiple

comparison test was used following Kruskal Wallis. A t-test was used to compare levels of neurotransmitters between PCPA and saline in the injection experiment. Paired t-test was used to compare values of papilla ratio for each animal at the start versus the end of the experiment. The monoamines and their metabolites were measured from the same brains for each animal so significant p-values were corrected with the sequential Dunn-Sidak adjustment to account for compounding of Type I error. Transformations of data are noted in results section if conducted.

Results

Implant experiment

Social conditions inhibitory to sex change

Dominant females implanted with PCPA or p-MPPI did not change sex; they had an average final papilla ratio not significantly different from initial values (paired t test for PCPA: t_4=0.99, p=0.38; initial=1.00±0.05; final=1.13±0.13; for p-MPPI: t_4=-0.38, p=0.72; initial=1.00±0.06; final=0.99±0.02). These females had distinct ovaries and did not develop an AGS. As expected, all control subordinate females whose brain monoamine levels were quantified had an average final papilla length/width ratio not significantly different from initial values (paired t test: t_9=0.80, p=0.45; initial=0.95±0.03; final=1.00±0.03). These females also had distinct ovaries and did not develop an AGS.

Social conditions permissive for sex change

Females that received sham and 5-HTP implants changed sex into males by the end of the experiment and their average final papilla ratio was significantly different from initial values (paired t test for sham: t_6=10.21, p<0.0001; initial=1.01±0.03; final=2.74±0.16; for 5-HTP: t_3=8.18, p=0.0038; initial=1.10±0.05; final=2.47±0.13). These females developed both testis and

AGS. As expected, control males had testis and AGS and retained male typical papillae; there was no significant difference between initial and final papilla ratios for control males (paired t test: t_5=2.35, p=0.065; initial=2.81±0.09; final=3.22±0.17).

Monoamine analysis

There was no significant difference (Table 4.2; Fig. 4.1.A) in whole brain 5-HT levels between treatments (ANOVA: $F_{5,31}$=1.30, p=0.29). There was a significant difference (Table 4.2; Fig. 4.1.B) in 5-HIAA levels between treatments (Kruskal Wallis: χ_5^2=16.56, p=0.0054; sequential Dunn-Sidak α_{adj} =0.0084). Animals treated with PCPA had 5-HIAA levels significantly lower than sham (Dunn's multiple comparison: Q=3.119, $Q_{critical}$=2.936, p<0.05) and males (Q=4.438, $Q_{critical}$=2.936, p<0.05). There was a significant difference (Table 4.2; Fig. 4.1.C) in 5-HIAA/5-HT ratio between treatments (ANOVA: $F_{5,31}$=5.81, p=0.0007; sequential Dunn-Sidak α_{adj} =0.0073). The 5-HIAA/5-HT ratio was higher in sham controls than in control females, p-MPPI, and PCPA treated animals (Tukey Kramer HSD p<0.05); 5-HIAA/5-HT ratio in males was higher than in PCPA treated animals (Tukey Kramer HSD p<0.05). There was no significant difference in DA (ANOVA: p=0.30, $F_{5,31}$=1.26), DOPAC (ANOVA: p=0.65, $F_{5,25}$=0.67), DOPAC/DA (ANOVA: p=0.66, $F_{5,25}$=0.66), or NE (ANOVA: p=0.76, $F_{5,31}$=0.52) across treatments (Table 4.2). DA, DOPAC and DOPAC/DA were natural log transformed to achieve normality.

There was no significant correlation between whole brain monoamine levels and rates of threat display, total aggression, displacements (natural log transformed) or courtship. For the correlation between monoamine levels and courtship rates we included only those animals that displayed courtship (control and 5-HTP groups). For rates of displacement received, we included only animals that remained female and were displaced (PCPA and p-MPPI). Rate of

displacement received was calculated as numbers of displacements received per minute of observation and averaged over the whole experimental period. There was a significant negative correlation between rate of displacement received (Fig. 4.3) and 5-HT levels ($p=0.040$, $r=-0.65$, $n=10$) and a trend for a negative correlation between rate of displacement received and 5-HIAA levels ($p=0.057$, $r=-0.62$, $n=10$). There was no significant correlation between any of the behaviors and whole brain monoamine levels of control females and males.

Injection experiments

PCPA injection

Of the ten animals that became dominant, seven received saline injections and three received PCPA injections. Papilla ratio did not change significantly for dominant (paired t test: $t_8=1.88$, $p=0.097$; initial, 1.07 ±0.04; final, 1.23±0.06) or subordinate animals (paired t test: $t_9=-0.04$, $p=0.972$; initial, 1.08 ±0.03; final, 1.08±0.05). Animals injected with PCPA had lower 5-HIAA ($t=4.76$, $df=16$, $p=0.0002$), 5-HT ($t=2.26$, $df=16$, $p=0.038$), and 5-HIAA/5-HT ($t=2.85$, $df=16$, $p=0.012$) than animals injected with saline vehicle (Table 4.3 and Fig. 4.2). When comparing animals that became subordinate versus dominant (Table 4.3 and Fig. 4.2), there were no significant differences in 5-HT ($t=0.21$, $df=16$, $p=0.833$), 5-HIAA ($t=0.09$, $df=16$, $p=0.927$), or 5-HT/5-HIAA ($t=0.003$, $df=16$, $p=0.998$). There were no significant differences in DA and NE levels between dominant and subordinate fish (Wilcoxon for NE: $Z=0.400$, $p= 0.689$; for DA: $Z=0.934$, $p=0.350$; Table 4.3) or between saline and PCPA treated fish (Wilcoxon for NE: $Z=-0.133$, $p= 0.894$; for DA: $Z=-0.845$, $p=0.398$).

Whether we considered all the animals together, or dominant animals alone, there were no correlations between any of the behaviors and whole brain monoamine levels. For subordinate fish, regardless of injection type, there was a significant positive correlation between rate of

approaches to the dominant and 5-HIAA levels (r=0.688, p=0.028, n=10) and between rate of approaches (Fig. 4.4) and 5-HIAA/5-HT (r=0.801, p=0.0054, n=10). The rate of approaches per minute was calculated for each daily observation and then averaged over the five days.

Fluoxetine injection

Of the ten fish that became dominant, seven received fluoxetine injections and three received saline injections. The papilla ratio of dominant fish was significantly more male-like at the end of the experiment relative to the beginning (paired t test: t_9=2.38, p=0.041; initial, 0.97 ±0.03; final, 1.13±0.08). The papilla of the subordinate fish did not change shape over the course of the experiment (paired t test: t_8=0.38, p=0.713; initial, 0.96 ±0.04; final, 0.97±0.03). There was no significant difference between fluoxetine and saline treated animals in the mean rate of any of the behaviors.

p-MPPI injection

Of the nine fish that became dominant, four were injected with saline and five with the 5-HT_{1A} receptor antagonist p-MPPI. The subordinate females did not show any significant change in papilla shape (paired t test: t_8=-1.176, p=0.273; initial, 1.02 ±0.02; final, 0.99±0.02) while the papilla of dominant fish became more male-like at the end of the experiment (paired t test: t_8=5.293, p=0.0007; initial, 0.95 ±0.03; final, 1.32±0.07). There was no significant difference between saline and p-MPPI treated animals in the mean rate of any of the behaviors

Discussion

Based on the existing literature in fishes, we predicted that male *L. dalli*, being the dominant individuals, would have lower serotonergic activity than females. The results from the implant experiment did not support this prediction, but rather showed a trend opposite to what we

expected: although the difference was not significant, females showed a trend for lower levels of 5-HT, 5-HIAA and 5-HIAA/5-HT than males.

The experiment on size-matched pairs showed that serotonergic activity does not affect social status or aggressive behavior in *L. dalli*. In fact, none of the injections manipulating 5-HT levels affected female aggression levels or the outcome of the fight. This contrasts with the studies on firemouth cichlid (*Cichlasoma meeki*) where a single i.p. injection of PCPA increased aggression towards a mirror (Adams et al., 1996), and on bluehead wrasse where a single i.p. injection of fluoxetine decreased aggression towards an intruder presented in a glass container (Perreault et al., 2003). An important difference is that in those studies the fish could not physically interact. The lack of social resolution has been shown to have an impact in fish at least in terms of hormonal response. In male cichlids, aggressive behavior against their mirror image was not sufficient to increase androgen levels, which typically occurs following actual fights (Oliveira et al., 2005). We know that in *L. dalli* physical interactions between individuals are important because the mere presence of a male behind a divider is not sufficient to inhibit sex change in the dominant female (Lorenzi et al., 2006). Low serotonergic function may predispose aggression before the fight (Summers et al., 2005; Summers and Winberg, 2006) but its effect in determining the outcome of the fight can be overcome by matching the fish with an opponent that is superior in term of size or other characteristics. Therefore pharmacological manipulations of the serotonergic system might show their effect more readily if the animals cannot physically interact, or if they are size-matched. In the study on wrasse cited above (Perreault et al., 2003), the fish do interact in the chronic treatment with fluoxetine and there is decreased aggression in the treated fish. However, in that study they used the intruder-resident paradigm, while we matched pairs of fish in a novel tank. Holding a territory is known to affect the outcome of fights

and subsequent social status in a wide variety of organisms (Hsu et al., 2006), suggesting that the use of different social contexts could explain some of the variation in the relationship between serotonergic function, aggression, and status.

On the other hand, there are other studies that, like ours, did not find an effect of serotonergic manipulations on aggression. For example, i.p. injection of a 5-HT_{1A} antagonist into resident mice increased some threat behavior towards the intruder but did not affect the number of escalated aggressive acts such as chases and bite attacks (Bell et al., 1996). In Siamese Fighting fish, *Betta splendens*, a single injection of a 5-HT_{1A} antagonist, 3 daily injections of PCPA, or chronic fluoxetine for 14 days had no significant effects on aggression against a mirror (Clotfelter et al., 2007). In the same study though, a single injection of 5-HT and of a 5-HT_{1A} agonist caused a reduction in aggression.

We found no differences in monoamine levels between dominant and subordinate animals in the size-matched pairs of fish. This contrasts results found in rainbow trout (Winberg and Lepage, 1998) and juvenile arctic charr (Winberg et al., 1991). There is the possibility that the difference in monoamine levels between dominant and subordinate animals is specific to certain regions of the brain (Summers et al., 2005) and that we missed it because we analyzed whole brains. A study on the bicolor damselfish *P. partitus* (Winberg et al., 1996) provides some evidence against this argument. They let pairs interact during a daily observation session for 5 days, and found no difference between dominant and subordinate individuals in 5-HT, 5-HIAA or 5-HIAA/5HT in any brain region (brainstem, hypothalamus, telencephalon). Also, similar to our results, Winberg and colleagues (1996) found that behavioral correlations were different depending on the social status. In subordinate *P. partitus* there was a significant positive correlation between 5-HIAA/5-HT ratio in the telencephalon and aggressive acts received. While

in dominant animals, the positive correlation was between 5-HIAA/5-HT ratio in the hypothalamus and aggressive acts performed (Winberg et al., 1996). In our PCPA injection experiment, we found that subordinate animals, but not dominant ones, have brain 5-HIAA/5-HT that correlate positively with rate of approaches given. The behavior of a subordinate approaching the dominant could be interpreted as a form of inspection or challenge. Therefore, higher serotonergic activity in the subordinate may be associated with less stability in social rank. In the implant experiment, females treated with 5-HT synthesis inhibitor PCPA and 5-HT_{1A} receptor antagonist p-MPPI in an inhibitory social environment, showed a significant negative correlation between rate of displacement received and 5-HT levels, and a trend for the negative correlation with 5-HIAA levels. Thus, the more the fish were displaced, the lower their 5-HT levels. This contrasts the work on other fishes included salmonids (Winberg et al., 1991) but fits with the fact that male *L. dalli* show a trend for higher serotonergic activity than females because dominant males are never displaced.

L. dalli is a very social species that lives at high densities (Behrents, 1983), and once a stable social hierarchy is established, it shows more ritualized behavior and less overt aggression. It is possible that, in a stable social environment, differences in monoamine levels between dominant and subordinate animals might not be as evident relative to situations in which the social hierarchy is being established, and more overt aggression is performed. The serotonergic system is closely associated with activation of the stress axis (Hypothalamus-Pituitary-Interrenal axis in fish) (Bell et al., 2007; Carpenter et al., 2007; Summers and Winberg, 2006) so the effect of a fight on the serotonergic system of the subordinate might be more pronounced in other species of fish because being confined to a tank with a dominant aggressive animal could be more stressful than for *L. dalli*. For example, in pairs of artic charr differences between

subordinate and dominant animals were detected even after 21 days of interactions (Winberg and Nilsson, 1993b). In that study, 5-HIAA and 5-HIAA/5-HT ratio in subordinates increased after 1 day of social interaction and remained significantly higher than dominant fish until day 21 in telencephalon and brain stem. In that experiment, the subordinate animals seemed to be stressed because after hierarchy establishment on day 1, they took a position close to the surface or in a corner and lost weight. In our laboratory tanks *L. dalli* establishes a stable social hierarchy similar to what it would experience in the wild and shows normal social and feeding behaviors so being confined to the tank does not appear to be stressful to subordinate fish. Perhaps this could explain the lack of differences in serotonergic function between dominant and subordinate individuals in *L. dalli*. These ideas are consistent with a meta-analysis on primates showing that subordinates have higher cortisol levels only in species where their rank was associated with high physical or psychological stress (Abbott et al., 2003).

None of our pharmacological manipulations affected sex change, and this contrasts with the study on *T. duperrey*, where an implant containing the SSRI sertraline inhibited sex change in a large female in the presence of a smaller female (Larson et al., 2003a). Larson and colleagues did not quantify behavior so it is unclear whether the treatment caused subordination and therefore inhibited sex change or whether it had only a peripheral effect in the gonad. They administered a different SSRI than we used in the present study, and there also was a difference in interaction time. They allowed fish to interact for 8 weeks after sertraline treatment, while our fish interacted only for 5 days after fluoxetine injection. We chose not to use an SSRI to increase serotonergic function in the 3 week implant experiment because SSRIs have been shown to have other effects such as increasing levels of neurosteroids in the brain and plasma (van Broekhoven and Verkes, 2003) opening up the possibility for non-specific or second order effects of the

manipulations. In our implant experiment, we analyzed the brains 3 weeks after male removal so we cannot exclude the possibility that some monoaminergic changes might take place at the very initial steps of sex change. Females of saddleback wrasse have been analyzed at different times during sex change in pairs (Larson et al., 2003b): they found that 5-HIAA/5-HT in the POA, tuberal nucleus and dorsomedial zone were lowest in the first 3 days of interaction, and then increased and stayed elevated; while 5-HIAA/5-HT in the raphe nucleus was high at the beginning and then decreased. We are aware that some of the changes in brain serotonergic activity produced by our implants were modest, but the small size of the fish, limited implant size and thus final dosage. Therefore, there is still the possibility that higher doses could have had an impact on sex change, but they may not be physiologically relevant.

We predicted that the serotonergic system would inhibit sex change, so we increased serotonergic activity in females in situations permissive to sex change. Unexpectedly, we found that female *L. dalli* show a trend for lower serotonergic function than males, so it makes sense that the manipulations did not have an effect on sex change because higher serotonergic function seems to be associated with male status. To exclude the possibility that in this species 5-HT might facilitate rather than inhibit sex change, we gave a PCPA implant to 4 dominant females in social groups permissive to sex change: after 3 weeks from male removal, the focal female had low brain 5-HIAA levels but nevertheless changed sex into male (Lorenzi unpublished data). Since these treatments did not have any effect we did not further pursue the hypothesis that serotonin might facilitate sex change.

Regarding sex differences, our result that female have lower levels contrasts with a study in rats that showed females having higher whole brain 5-HT and 5-HIAA levels than males (Carlsson et al., 1985). To our knowledge, there are not many studies looking at sex differences

in serotonergic activity in fish, but in the bicolor damselfish (*Pomacentrus partitus*) there was no effect of sex on serotonergic activity when comparing dominant and subordinate animals (Winberg et al., 1996). It is interesting to note that in tilapia, *O. mossambicus*, decreasing 5-HT brain levels produces a female biased sex ratio (Tsai et al., 2000). Even if this applies only during sexual differentiation, the female condition in this case seems to be associated with low 5-HT levels, and this is consistent with our findings.

From the present study, it is clear that our serotonin manipulations were not sufficient to control sex change but we cannot exclude that the possibility that our doses were not high enough or that serotonin acts synergistically with other neurotransmitters or peptides to regulate sex change during adulthood. We did not see any significant difference in DA, DOPAC or NE among individuals so it is unlikely that any of these neurotransmitters alone, including serotonin, plays a major role. Also, further studies will need to test whether changes at the levels of the receptors rather than absolute levels of the transmitter are important to regulate sex change. There is evidence that receptors might play a role in modulating aggression levels. In rodents, aggressive strains have higher 5-HT_{1A} autoreceptor sensitivity than docile strains (Caramaschi et al., 2007), and non-aggressive individuals show a greater level of expression of 5-HT_{1A} receptors in the midbrain (Popova et al., 2007).

In summary, direct comparisons across studies is difficult because of substantial between study variation in type of pharmacological manipulation, season and reproductive stage of the animals, experimental protocol, and social context. However, the results from ours and other studies suggest that the role of the serotonergic system in the modulation of aggressive behavior can vary widely in species with different social systems or in different social settings within a species (pairs vs. larger social groups). Thus, analyses that systematically alter these parameters

may be needed to evaluate the general applicability of the association among low serotonergic function, aggression, social dominance and sex change.

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Table 4.1: Summary of treatments for the implant experiment and predictions based on the hypothesis that high serotonergic function inhibits sex change.

Treatment	Social environment	Serotonergic function	Expected sex change?
pMPPI (5-HT1A antagonist)	Non-permissive	Decrease	Yes
PCPA (5-HT synthesis inhibitor)	Non-permissive	Decrease	Yes
5-HTP (5-HT precursor)	Permissive	Increase	No
Sham (wax only)	Permissive	No effect	Yes

Table 4.2: Monoamine levels in the different treatments.

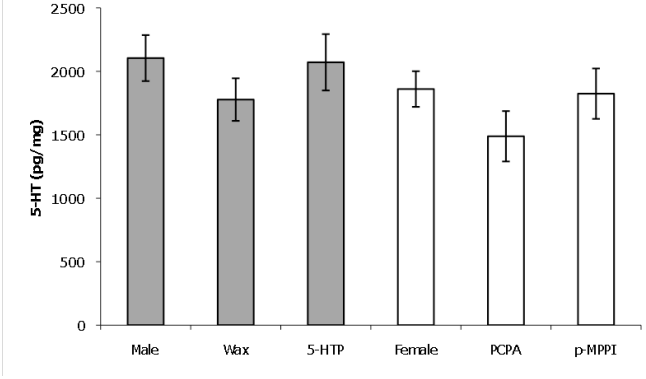
	Male			Female		
	Male	**Wax**	**5-HTP**	**Female**	**PCPA**	**p-MPPI**
5-HT	2104.52±181.23 (6)	1777.59±167.79 (7)	2071.38±221.96 (4)	1860.56±140.38 (10)	1488.42±198.53 (5)	1823.96±198.53 (5)
5-HIAA	601.99±63.68 (6)	574.14±58.96 (7)	597.01±77.99 (4)	414.49±49.33 (10)	255.88±69.76 (5)	383.39±69.76 (5)
5-HIAA/5-HT	0.294±0.024 (6)	0.322±0.022 (7)	0.276±0.029 (4)	0.222±0.019 (10)	0.169±0.026 (5)	0.210±0.026 (5)
DA	219.27±25.51 (6)	207.90±23.61 (7)	217.68±31.23 (4)	266.58±19.76 (10)	245.11±27.94 (5)	205.87±27.94 (5)
DOPAC	59.88±20.78 (5)	44.31±12.16 (5)	49.60±14.70 (3)	65.84±17.89 (9)	73.64±18.53 (5)	71.68±13.62 (4)
DA/DOPAC	0.252±0.078 (5)	0.226±0.078 (5)	0.270±0.101 (3)	0.263±0.058 (9)	0.288±0.078 (5)	0.368±0.088 (4)
NE	2592.41±245.86 (6)	2141.85±229.69 (7)	2150.60±361.14 (4)	2310.13±184.41 (10)	2343.86±129.84 (5)	2305.21±129.18 (5)

Values are mean±standard error in pg/mg of fresh brain tissue. The number in parenthesis represents sample size.

Table 4.3: Values are mean ± standard error in pg/mg of fresh brain tissue.

	Saline injection (N=8)	**PCPA injection** (N=10)	**Dominant** (N=8)	**Subordinate** (N=10)
5-HT	629.50±62.35	474.50±35.94	552.73±74.81	535.91±37.44
5-HIAA	300.00±26.30	162.38±15.13	225.88±31.17	221.68±31.45
5-HIAA/5-HT	0.49±0.04	0.35±0.03	0.41±0.04	0.41±0.04
DA	142.88±15.52	146.70±8.99	151.13±14.41	140.10±9.81
NE	3539.38±159.87	3808.70±287.79	3735.88±269.41	3651.50±238.31

A)



B)

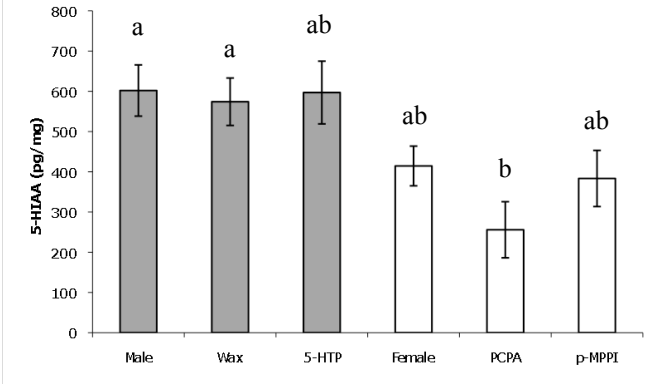
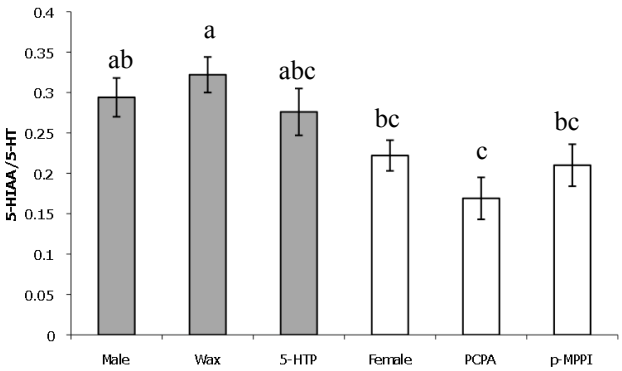


Figure 4.1: Mean brain levels of 5-HT (A), 5-HIAA (B), 5-HIAA/5-HT (C) in animals that received implants and non-manipulated males and females. Bars represent standard errors. Gray bars are associated with treatments where the animals are males at the end of the experiment, and white bars with animals that are females. Different letters indicate significant difference ($p<0.05$).

Figure 4.1: Continued

C)



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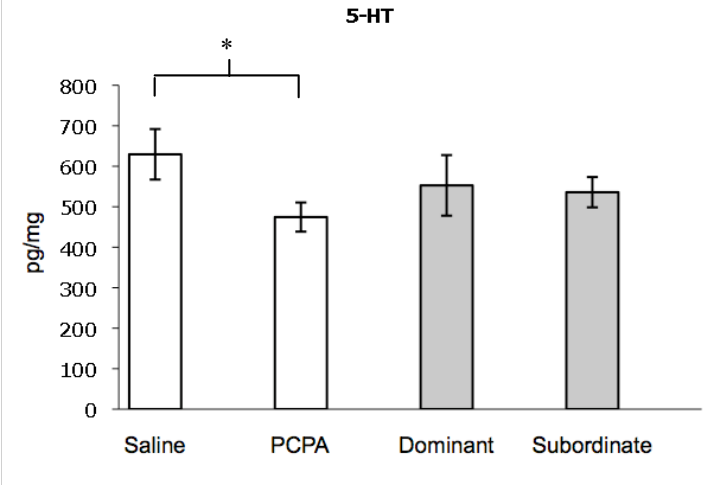

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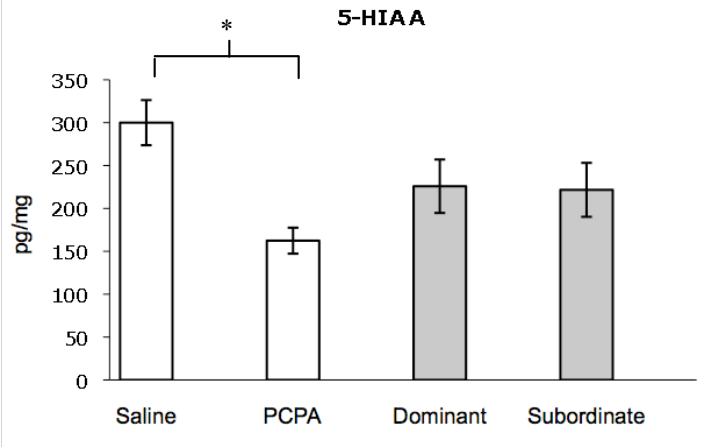
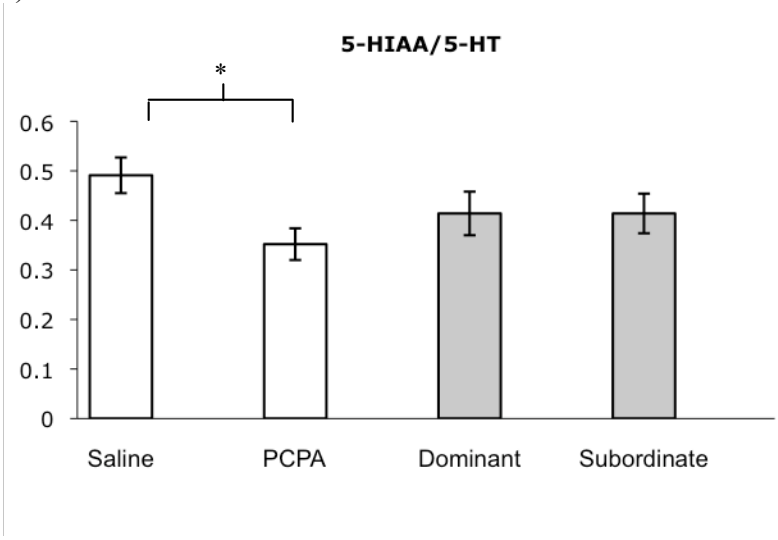


Figure 4.2: Comparison of mean brain levels of 5-HT (A), 5-HIAA (B), and 5-HIAA/5-HT (C) in size-matched pairs of animals that received either PCPA or saline injections. White columns represent the comparison based on injection received, while gray columns represent the comparison based on social status. Values are measured in pg/mg of fresh tissue. Bars represent standard errors. The asterisk indicates significant difference ($p<0.05$).

Figure 4.2: Continued

C)



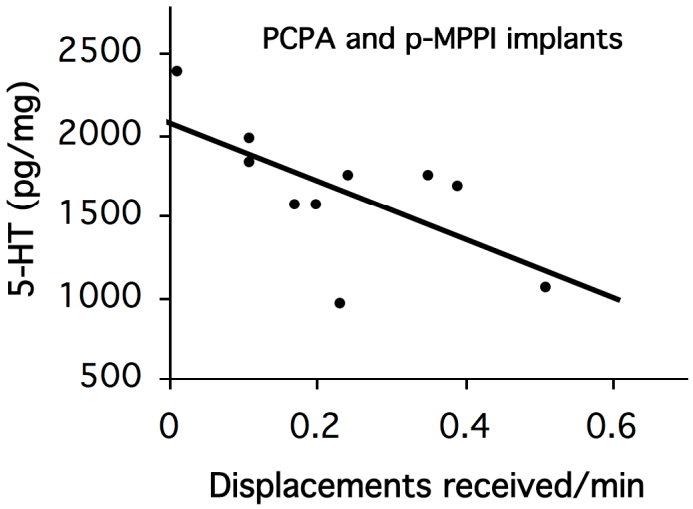


Figure 4.3: Correlation between 5-HT brain levels (pg/mg of fresh tissue) for fish that received PCPA and pMPPI implants and rate of displacements received/minute of behavioral observation (R^2=0.428; p=0.0403; F=5.98, N=10).

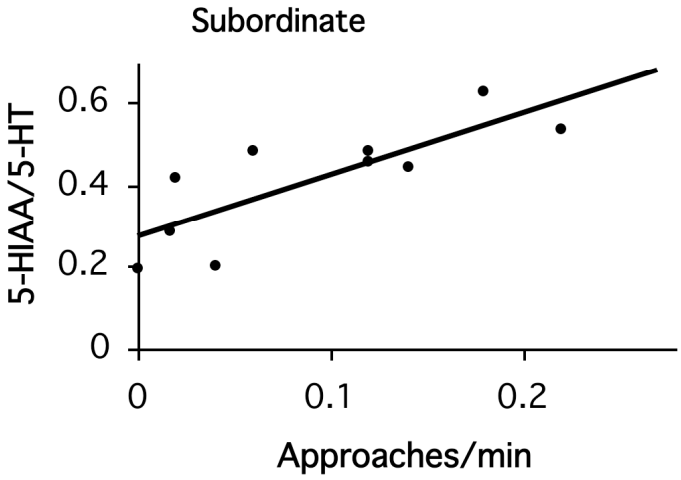


Figure 4.4: Correlation between brain serotonergic activity and rate of approaches/minute for fish that became subordinate after injection of either PCPA or saline (R^2=0.641; p=0.0054; F=14.28).